MODULATION OF ATP-SENSITIVE POTASSIUM CHANNELS BY HYDROGEN SULFIDE AND HYDROXYLAMINE

A Thesis

Submitted to the College of Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy in Physiology

University of Saskatchewan

by

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ABSTRACT

ATP-sensitive potassium (K^+) channels (K_{ATP}) in vascular smooth muscle cells (VSMC) play a major role in the regulation of vascular tone by coupling cell contractility and K^+ fluxes to cellular metabolism. They are composed of the regulatory sulphonylurea receptors (SUR) and the pore-forming inwardly rectifying K^+ (Kir) channels. SUR subunits interact closely with Kir subunits by conferring their sensitivity to nucleotide or sulphonylurea. However, the modulatory mechanisms of K_{ATP} channels in VSMC are largely unknown. In particular, the effects of hydrogen sulfide (H_2S) and hydroxylamine (HA) on K_{ATP} channels and underlying mechanisms have not been addressed in VSMC of resistance arteries. The combined approaches including molecular biology, biochemical assays, and patch-clamp techniques were applied. The electrophysiological and pharmacological features of native K_{ATP} channels in VSMC and cloned K_{ATP} channels in HEK-293 cells, and the modulation of K_{ATP} channels by H_2S and HA in single freshly isolated VSMC from rat mesenteric arteries were characterized. In the present study, only small conductance K_{ATP} channels of 13 pS were found in rat mesenteric artery VSMC. The recorded macroscopic and unitary K_{ATP} currents were activated by nucleoside diphosphate in the presence of magnesium and K^+ channel openers, inhibited by a specific K_{ATP} channel blocker glibenclamide, but were insensitive to ATP inhibition. The reversal potential shifted rightward in response to the elevation of extracellular K^+ and matched the calculated K^+ equilibrium potential, indicating the basal currents in both VSMC and HEK-293 cells are carried by K^+ ions. Heterologous expression of Kir6.1 with SUR2B in HEK-293 cells formed functional channels and elicited whole-cell K^+ currents, which shared some similar biophysical

characteristics of native K_{ATP} channels in VSMC. Basal K_{ATP} currents and resting membrane potential in VSMC were reduced by glibenclamide, demonstrating that K_{ATP} channels contribute to background K^+ conductance and in the setting of resting membrane potential in this resistance artery. Exogenous H_2S enhanced macroscopic and unitary K_{ATP} currents with an EC_{50} of 116 ± 8.3 μM and hyperpolarized membrane potential. H_2S activated K_{ATP} channels by increasing the open probability of single channels, but not single channel conductance. The reduced endogenous H_2S production by D, L-propargylglycine resulted in the attenuation of K_{ATP} currents. H_2S-induced activation of K_{ATP} channels and resultant hyperpolarization were not mediated by cGMP signaling pathway. HA enhanced reversibly K_{ATP} currents in a dose-dependent fashion with an EC_{50} of 54±3.4 μM and also hyperpolarized the cell membrane. HA-stimulated K_{ATP} currents were blocked by free radical scavengers (superoxide dismutase and N-acetyl-L-cysteine), and K_{ATP} channels were stimulated by a free radical generating system (hypoxanthine/xanthine oxidase), indicating the involvement of superoxide (O_2^-) in HA effects. Sodium nitroprusside and 8-Br-cGMP did not affect basal K_{ATP} currents and HA-stimulated K_{ATP} currents, disproving the involvement of NO-sGC-cGMP-mediated signaling pathway in the HA effects. Therefore, HA-induced K_{ATP} channel activation and hyperpolarization are likely due to the generation of O_2^-. In conclusion, K_{ATP} channels in resistance artery VSMC serve as the regulatory targets of H_2S and HA. These two endogenous molecules modulate K_{ATP} channels via different mechanisms. H_2S may directly act on K_{ATP} channel proteins while HA oxidized them via the formation of O_2^-, leading to the activation of K_{ATP} channels.

ACKNOWLEDGEMENTS

First and foremost, I am sincerely grateful to my supervisor Dr. Rui Wang. Thanks to Dr. Wang's inspiration and guidance, I was sculpted from barely knowing how to operate a pipette in 1997 into becoming fully capable and wholly independent in critically analyzing scientific literature, designing and executing experimental strategies, and writing, presenting, and publishing my results. Dr. Wang's commitment to work hard and strive for excellence has been a constant source of inspiration to encourage me to pursue my graduate studies. In addition, I would like to thank Dr. Wang for having the confidence in me, and allowing me to pursue my own research interests. I especially thank Dr. Wang for supporting my external studentship applications and my attendance at several scientific conferences. All in all, Dr. Wang's whole-hearted support, encouragement, and friendship in all of my endeavors was, and remains to be, invaluable to me.

I would like to offer special thanks to Dr. V. Gopalakrishnan and Dr. J. Thornhill for their guidance and advice that were of tremendous value in helping me complete my thesis work. I am much indebted to Dr. Gopalakrishnan for giving me a great chance to do the collaborative research project under his guidance. I acknowledge and sincerely thank other members of my thesis advisory committee, Dr. T. Fisher, Dr. A. Wollin and Dr. W. Walz for their guidance, support, and friendship throughout my graduate studies. Dr. P. Sulakhe and Dr. N. West are gratituded for their chairing the advisory committee meetings for several years.

I particularly thank Dr. Rémy Sauvé for agreeing to serve as my external examiner and for providing excellent insights into my work. I am honored to have had

such a renowned member of membrane ion channel scientific community serve on my thesis defense committee.

I also want to thank the following organizations for provoding me scholarship and studentship awards to complete my graduate studies, such as the Heart and Stroke Foundation of Canada (HSFC), the Canadian Hypertension Society (CHS) in partnership with the Canadian Institute of Health Research (CIHR) or the Medical Research Council of Canada, the Pharmaceutical Manufactures Association of Canada, and Pfizer Inc. I am also grateful for numerous travel awards that enabled me to present my research work at many scientific conferences from HSFC, CIHR, CHS, the Canadian Physiological Society, the American Society for Pharmacology and Experimental Therapeutics, the College of Medicine, the College of Graduate Studies and Research, the University of Saskatchewan Vice-President office (Academic & Student Affairs), and Merck-Frosst Canada & Co. The work in this thesis was supported by a grant from the CIHR and the Natural Sciences and Engineering Research Council of Canada to Dr. R. Wang.

I express my gratitude to Dr. L. Wu, Dr. C Xu, and Mr. Y. Lu for their invaluable support on countless occasions. Additionally, I would like to thank Dr. K. Cao for his great help for cell transfection. Finally and most importantly, I extend my sincere thanks to my family and all of my friends for their invaluable support in keeping a balance in my life throughout these past several years.

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LIST OF ABBREVIATIONS

ADP	–	Adenosine diphosphate
4-AP	–	4-aminopyridine
ATP	–	Adenosine triphosphate
Ba^{2+}	–	Barium
8-Br-cGMP	–	8-bromo-cGMP
CBS	–	Cystathionine β-synthase
cGMP	–	3'5'-cyclic guanosine monophosphate
ChTX	–	Charybdotoxin
CLT	–	Chloramine T
CO	–	Carbon monoxide
COS-7	–	African green monkey cells
CSE	–	Cystathionine γ-lyase
DTBNP	–	2, 2'-dithio-bis(5-nitropyridine)
DTT	–	D, L-dithiothreitol
DTNB	–	5, 5'-dithio-bis(2-nitrobenzoic acid)
EC_{50}	–	Half excitatory concentration
FBS	–	Fetal bovine serum
GDP	–	Guanosine 5'-diphosphate
GSH	–	Reduced glutathione
GSSG	–	Oxidized glutathione
HA	–	Hydroxylamine
HEK-293	–	Human embryonic kidney cells

H_2O_2	–	Hydrogen peroxide
HS^-	–	Thiolate anion
H_2S	–	Hydrogen sulfide
HX	–	Hypoxanthine
IbTX	–	Iberiotoxin
IC_{50}	–	Half inhibitory concentration
K_{ATP}	–	ATP-sensitive K^+ channels
K_{Ca}	–	Ca^{2+}-activated K^+ channels
KCOs	–	K^+ channel openers
$[K^+]_i$	–	Intracellular K^+ concentration
Kir	–	Inward rectifier K^+ channels
K_{NDP}	–	NDP-dependent K^+ channels
$[K^+]_o$	–	Extracellular K^+ concentration
K_V	–	Voltage-dependent K^+ channels
MAB	–	Mesenteric artery bed
MB	–	Methylene blue
Mg^{2+}	–	Magnesium
NAC	–	N-acetyl-L-cysteine
NDP	–	Nucleoside diphosphate
NEM	–	N-ethylmaleimide
NO	–	Nitric oxide
NOS	–	NO synthase
O_2^-	–	Superoxide anion

OH^-	–	Hydroxyl radical
$OONO^-$	–	Peroxynitrite
p-CMPS	–	p-chloromercuri-phenylsulfonic acid
4-PDS	–	4, 4'-dithiodipyridine
PHE	–	Phenylephrine
p-HMPS	–	p-hydroxymercuri-phenylsulfonic acid
PKG	–	cGMP-dependent protein kinase G
PMB	–	4-hydroxy-mercuribenzoic acid
PPG	–	D, L-propargylglycine
PTA	–	Phosphotungstic acid
RNS	–	Reactive nitrogen species
ROS	–	Reactive oxygen species
RS	–	Thiyl radical
–SH	–	Sulphydryl or thiol group
sGC	–	Soluble guanylyl cyclase
SMC	–	Smooth muscle cells
SNP	–	Sodium nitroprusside
SNAP	–	S-nitroso-N-acetyl-penicillamine
SOD	–	Superoxide dismutase
S–S	–	Disulfide bond
SUR	–	Sulphonylurea receptor
TEA	–	Tetraethylammonium chloride
TEMPO	–	Tetramethylpiperidine-N-oxyl

Thimerosal	–	[(o-carboxyphenyl)thio] ethylmercury sodium
Tiron	–	4, 5-dihydroxy-1, 3-benzene disulfonic acid
VDCC	–	Voltage-dependent Ca^{2+} channels
VSMC	–	Vascular smooth muscle cells
X	–	Xanthine
XO	–	Xanthine oxidase

1. INTRODUCTION

1.1 Gasotransmitters

1.1.1 Definition and family members of gasotransmitters

Gasotransmitters are newly termed as small signalling molecules of endogenous gases with physiological importance (Wang, 2002, 2004). They play a major role in physiological and pathological processes such as blood pressure regulation, neurotransmission release, inflammatory processes, etc. Following the identification of nitric oxide (NO) and carbon monoxide (CO) as gasotransmitters, hydrogen sulfide (H_2S) may be qualified as the third one. The criteria to define gasotransmitters include:

a) They are small molecules of gas, like NO and CO.

b) They are freely permeable to membranes. As such, their cellular effects will not rely on cognate membrane receptors.

c) They are endogenously and enzymatically generated and their generation is regulated.

d) They have well-defined specific functions at physiologically relevant concentrations. Thus, manipulating the endogenous levels of these gases evokes specific physiological changes. For instance, NO and CO both participate in vasorelaxation and synaptic transmission in the central nervous system.

e) Their functions can be mimicked by their exogenously applied counterparts.

f) Their cellular effects may or may not be mediated by second messengers, but should have specific cellular or molecular targets. For instance, NO and CO activate K_{Ca} channels in the plasma membranes either directly or are mediated by the cGMP pathway.

According to the above criteria, the gasotransmitter family may consist of many yet unknown endogenous gaseous bio-molecules, such as ammonia (NH_3), formaldehyde (CH_2O), acetaldehyde (CH_2CH_2O), and ethylene (CH_2CH_2), etc. (Wang, 2002, 2003). In this thesis, the effects of H_2S and NO, and their donors on K_{ATP} channels will be studied and discussed.

1.1.2 Modulation of K^+ channels by gasotransmitters in VSMC

Gasotransmitters are important endogenous signalling molecules and share common chemical features and biological action modes. Among many cellular and molecular targets of gasotransmitters, membrane ion channels, especially potassium (K^+) channels, are the key signal transduction link. The regulation of K^+ channels by gasotransmitters can result from the activation of different second messengers or the direct chemical modifications between gasotransmitters and channel proteins. This direct interaction between K^+ channels and gasotransmitters exhibits the following characteristics:

1) Direct modification of K^+ channels by gasotransmitters is independent of membrane receptor and conventional second messengers, representing a novel class of signal transduction mechanism. This direct modulation of K^+ channels by gasotransmitters has been demonstrated in many cases for NO (Bolotina *et al.*, 1994; Wu

et al., 2002; Liu *et al.*, 2002), CO (Wang & Wu, 1997; Kaide *et al.*, 2001; Wu *et al.*, 2002; Jaggar *et al.*, 2002;), and H_2S (Zhao *et al.*, 2001).

2) K^+ channels on plasma membranes play important roles in the regulation of cellular functions, which are complicated by multiple members of K^+ channel families. Furthermore, K^+ channels themselves serve as important signal transduction links by transducting specific K^+ ions and directly coupling to diverse biological functions.

3) The modulation and mobilization of classic second messengers by gasotransmitters has been a hot topic, although the effects of gasotransmitters on K^+ channel activity may or may not be mediated by second messengers.

Gasotransmitters interact directly with K^+ channels in three specific modes of chemical modification. NO covalently modifies free cysteine residues in proteins via S-nitrosylation (Stamler *et al.*, 1992; Stamler 1994). The S-nitrosylation of K_{Ca} channel proteins (â-subunit) by NO would directly change the functional activity of these channels (Bolotina *et al.*, 1994; Liu *et al.*, 2002; Wu *et al.*, 2002). Direct interaction of gasotransmitters with K^+ channel proteins also applied in the case of CO. Many reported effects of CO on K_{Ca} channels are not regulated by known second messengers. Chemical modification of histidine residues of K_{Ca} channel proteins (á-subunit) by CO via the formation of hydrogen bond, a process of carboxylations, has been indicated (Wang & Wu, 1997; Wang *et al.*, 1997; Wu *et al.*, 2002). Direct modulation of K_{ATP} channels by H_2S is not mediated by cGMP or other known second messengers (Zhao *et al.*, 2001). A chemical interaction of H_2S with cysteine residues, including –SH group and its disulfide, of ion channel protein is hypothesized. The formation of adduct of HS^- with free –SH groups, a thiolation mechanism, or the breakdown of disulfide bonds, a dethiolation mechanism, by H_2S are alterative molecular mechanisms to be determined.

Therefore, the direct chemical modification of amino acid residues, such as cysteine and histidine, via S-nitrosylation, carboxylation, and thiolation/dethiolation, provides the molecular mechanisms of direct interaction of NO, CO, and H_2S with K^+ channel proteins, respectively.

1.2 K_{ATP} channels in VSMC

1.2.1 Function and significance of K_{ATP} channels

K^+ channels are a specific class of membrane proteins and have been found in all organisms and various types of cells including VSMC. They play an essential role in the physiological regulation of vascular tone and blood flow, stabilization of membrane potential, release of hormones or transmitters, and control of cell volume, etc. K^+ channel opening or closing can be regulated by different stimuli such as a change in cell membrane potential and small active molecules called ligands. At least four classifications of K^+ channels were identified in VSMC, including voltage-dependent K^+ (Kv) channels, Ca^{2+}-activated K^+ (K_{Ca}) channels, ATP-sensitive K^+ (K_{ATP}) channels, and inward-rectifier K^+ (K_{IR}) channels (Nelson & Quayle, 1995; Standen & Quayle, 1998). Thus, the opening of K^+ channels by vasodilators in VSMC increases K^+ efflux, which causes membrane hyperpolarization. This closes voltage-dependent Ca^{2+} channels, decreasing Ca^{2+} entry, which leads to vasodilation. In contrast, inhibition of K^+ channels may contribute to vasoconstriction or vasospasm as well as compromise the ability of an artery to dilate (Nelson & Quayle, 1995) (Fig. 1). The ability of blood vessel smooth muscle to constrict is affected by the change in $[Ca^{2+}]_i$, which is largely controlled by the

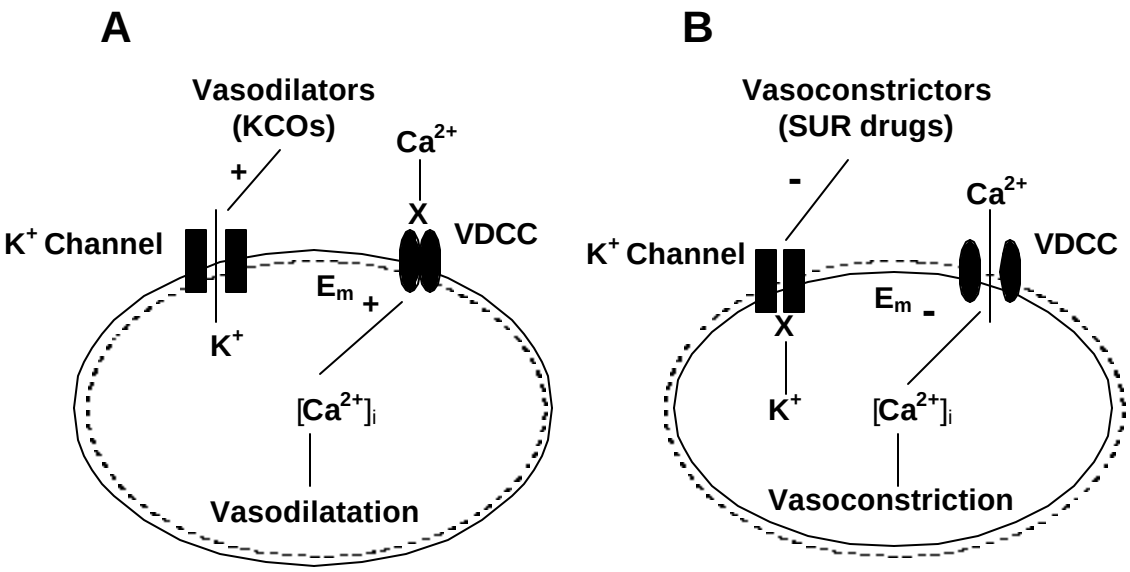


Fig. 1: Schematic illustration of the key events involved in the vascular smooth muscle in response to K^+ channel vasodilator or vasoconstrictor. **A.** Activation of K^+ channel in cell membrane allows K^+ efflux, causing an increase in membrane potential (E_m) (hyperpolarization) and consequent inhibition of voltage-dependent Ca^{2+} channels (VDCC) and a decrease in $[Ca^{2+}]_i$ level, resulting in vascular muscle relaxation or vasodilatation. **B.** In the reverse case, inhibition of vascular muscle K^+ channel decreases K^+ efflux and hence, decreases E_m (depolarization). VDCC channels will open in response to the decreased E_m (depolarization), allowing Ca^{2+} to enter the cell and to increase the level of $[Ca^{2+}]_i$, resulting in vascular contraction or vasoconstriction. KCOs: K^+ channel openers, SUR drugs: sulfonylurea drugs. Vasoconstrictors include: angiotensin II, endothelin, vasopressin, noradrenaline, histamine, serotonin, neuropeptide etc.; while vasodilators contain: CGRP, adenosine, isoprenaline, prostacyclin, vasoactive intestinal peptide, gasotransmitters etc.

membrane potential. As a major regulator of membrane potential in VSMC, K^+ channel activity is therefore an important determinant of vascular tone, arterial diameter, peripheral resistance, and blood pressure. K_{ATP} channel activity increased during ischemia, hypoxia and metabolic inhibition. The hallmark of K_{ATP} channels is that their activities are inhibited by intracellular ATP and activated by intracellular nucleoside diphosphates (NDP) in the presence of Mg^{2+}. Both ATP and ADP are energy molecules generated in the cellular metabolism. Thus, K_{ATP} channels couple the cellular metabolic state to the electrical activity of cell membrane and play an important role in the regulation of cellular functions under physiological and pathophysiological status. Under physiological conditions, metabolic regulation of K_{ATP} channel is achieved through changes in the cytosolic ratios of [ATP]/[ADP]. K_{ATP} channels appear to be tonically active in some vascular beds like the mesenteric artery bed and contribute to the physiological regulation of vascular tone and blood flow (Nelson & Quayle, 1995). Under pathophysiological conditions or disease states, such as hypoxia, ischemia, acidosis, septic shock, hypertension, and diabetes, K_{ATP} channels may play an important role in the regulation of tissue perfusion and are important targets for therapeutic drugs like sulphonylurea drugs and K^+ channel openers (Brayden, 2002).

1.2.2 Electrophysiological and pharmacological features of K_{ATP} channels

K_{ATP} channels in VSMC have specific electrophysiological or pharmacological characteristics, in addition to common features in other tissues. These common features are summarized as follows: 1) K_{ATP} channel activity exhibits little or no voltage or time dependence. 2) The channels exhibit weak inward rectification on strong depolarization. 3) The channel opening appears in bursts, but flickering (brief openings and closings)

within bursts decreases when the membrane is depolarized strongly. 4) The channel activity is inhibited by glibenclamide and ATP, and activated by KCOs and MgNDP.

In VSMC, apart from above common features, K_{ATP} channels possess the following specific characteristics in electrophysiology and pharmacology: 1) Small or intermediate conductance K_{ATP} channels of 15-50 pS are predominant in symmetrical 145 mM [K^+] despite the presence of large-conductance channels of 100-258 pS. 2) MgNDP is a more important regulator for K_{ATP} channels of 20-25 pS than ATP. This is why vascular K_{ATP} channels are also called K_{NDP} channels. 3) Vascular K_{ATP} channels exhibit insensitivity to ATP inhibition, which is a unique feature of vascular K_{ATP} channels. Low concentrations of ATP (0.1-100 μM) facilitate the channel in its open state, while physiological concentrations of ATP (1-3 mM) does not inhibit channel activity (Yokoshiki *et al.*, 1997). Whether or not this insensitivity of vascular K_{ATP} channels to ATP inhibition determines channel activation in the resting state, and then contributes to the background K^+ conductance and sets the resting membrane potential in VSMC has not been made clear.

K_{ATP} channel activity can be modulated by different pharmacological agents such as KCOs and specific inhibitors like sulphonylurea drugs (Ashcroft & Ashcroft, 1992; Edwards & Weston, 1993). KCOs are a structurally unrelated and chemically diverse group of drugs with a broad spectrum of potential therapeutic applications to reduce cell excitability and correct hypertension and ischemia (Lawson, 1996). The most commonly studied KCOs include diazoxide, pinacidil, cromakalim, nicorandil, and minoxidil sulfate (Gopalakrishnan et al., 1993). In term of the interaction with Kir6.0 or SUR subunit, K_{ATP} channel inhibitors fall into two groups. Imidazolines and antimalarials (quinine and mefloquine) block K_{ATP} channels by binding to Kir6.2 (Mukai *et al.*, 1998;

Proks *et al.*, 1997; Gribble *et al.*, 2000); whereas sulphonylureas (tolbutamide, glibenclamide, etc.) and benzamido derivaties (meglitinide) close K_{ATP} channels by binding with high affinity to SUR. Sulphonylureas also interact with Kir6.2, but with low affinity (Gribble et al., 1997). All drugs that block K_{ATP} channels stimulate insulin secretion but only those that interact with SUR subunit are used therapeutically to treat type II (non-insulin-dependent) diabetes mellitus. The further affinity binding assays demonstrated that the low-affinity site for sulphonylurea is independent of SUR, as a similar block is seen when Kir6.2 is expressed in the absence of SUR (Gribble *et al.*, 1997, 1998); while the high-affinity site lies on SUR, as it is only present when SUR is co-expressed with Kir6.2. Two SUR genes have been identified, one of which encodes â-cells isoform (SUR1) and the other cardiomyocytes (SUR2A) and smooth muscle cell (SUR2B) isoforms of the sulphonylurea receptor (Aguilar-Bryan *et al.*, 1995; Inagaki *et al.*, 1996; Isomoto *et al.*, 1996). These isoforms account for the differential tissue selectivity to sulphonylureas. Tolbutamide blocks â-cells K_{ATP} channels with high affinity but has relatively little effect on cardiac myocyte channels, because SUR1 contains a high-affinity tolbutamide-binding site missing in SUR2A. However, glibenclamide is less tissue-specific, blocking both â-cells and cardiomyocyte K_{ATP} channels (Venkatesh *et al.*, 1991; Gribble *et al.*, 1998). This results from unique chemical structure of glibenclamide, which consists of the benzamido (meglitinide) moiety, in addition to the high-affinity sulphonylurea (tolbutamide) moiety. Meglitinide inhibits both â-cells and cardiomyocyte K_{ATP} channels by binding to SURs at a separate site. Glibenclamide photolabelling of the transmembrane domains (TMD) 1-5 segment of SUR1 is consistent with a complex-binding site in which the sulphonylurea moiety binds to TMD12-17 and the benzamido group is in close proximity to the TMD1-5

segment (Babenko *et al.*, 19999). Thus, glibenclamide, as the second generation sulphonylurea, is widely used as a common blocker of different K_{ATP} channels in â-cells, cardiomyocyte, and vascular smooth muscle cells.

1.2.3 Molecular basis of K_{ATP} channels in VSMC

The molecular cloning experiments revealed that the K_{ATP} channel is a heterooctamer assembly. It is composed of a pore-forming inwardly rectifying K^+ channel (Kir6.x) tetramer and a regulatory sulphonylurea receptor (SURx) tetramer (Saskura *et al.*, 1995; Lorenz *et al.*, 1998) (Fig. 2). Kir6.x consists of Kir6.1 and Kir6.2 interactions with SUR subunit (Proks *et al.*, 1999; Tucker *et al.*, 1997). As the pore-forming subunit of K_{ATP} channel complex, Kir6.1 and Kir6.2 dictates the K^+ selectivity, inward rectification and unitary conductance for K_{ATP} channels. Whether the expression of Kir6.1 or Kir6.2 alone can elicit functional K_{ATP} currents has been a matter of debate (Gribble *et al.*, 1997). SURx is composed of SUR1, SUR2A, and SUR2B, which belong to the ATP-binding cassette superfamily and confer sulphonylurea sensitivity (Standen *et al.*, 1989; Cook & Hales, 1984; Inagaki *et al.*, 1996). SURx has 17 putative transmembrane domains with an extracellular N-terminus and an intracellular C-terminus (Inagaki *et al.*, 1995b; Isomoto *et al.*, 1996; Raab-Graham *et al.*, 1999). Two nucleotide binding folds, NBF-1 and NBF-2, present on the cytoplasmic side are located in the loop between TMD_1 and TMD_2, TMD_2 and the C terminus. Thus, SUR subunits provide the binding site for endogenous modulators (ADP, ATP, and GDP) and exogenous compounds (KCOs and glibenclamide) (Ashcroft & Gribble, 2000). K_{ATP} channel complex is assembled with 1:1 tetrameric stoichiometry of Kir6.x and SURx subunits $(Kir6.x/SURx)_4$. Although Kir6.x and SURx subunits are structurally distinct,

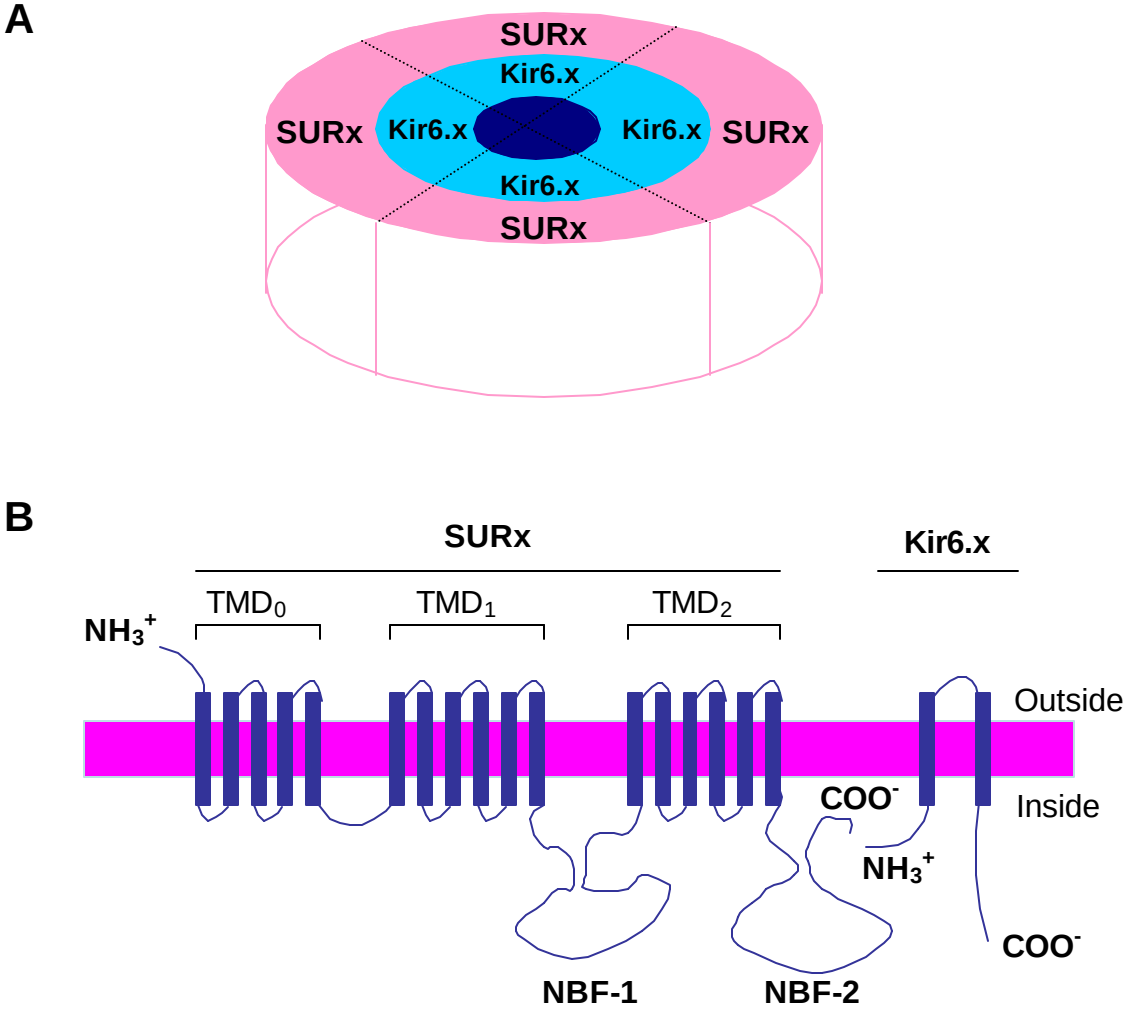


Fig. 2: Molecular structure and stoichiometry of K_{ATP} channel. A. Assembly of K_{ATP} channel. The K_{ATP} channel is a hetero-octamer composing of two subunits: the pore-forming subunit Kir6.x (Kir6.1 or Kir6.2) and the regulatory subunit sulphonylurea receptor SURx (SUR1, SUR2A, or SUR2B). **B.** Membrane topology of SURx and Kir6.x (modified from Fujita & Kurachi, 2000). The sulfonylurea receptor has been proposed to have three transmembrane-spanning regions (TMD_0, TMD_1, and TMD_2), each consisting of five, six, and six transmembrane domains, respectively. Two nucleotide binding folds (NBF-1 and NBF-2) on the cytoplasmic side are located in the loop between TMD_1 and TMD_2, and TMD_2 and the C terminus, respectively. Kir6.x has two transmembrane domains.

they have to physically interact with each other to constitute functional K_{ATP} channels (Lorenz *et al.*, 1998). and each has two transmembrane domains. Both C- and N-termini of Kir6.1 and Kir6.2 are located inside the cell and are important for intracellular ATP binding and

Most of our knowledge about the tissue-type-specific expression of different K_{ATP} channel subunits is derived from the detection of the transcript of these subunits and from the pharmacological sensitivity of native K_{ATP} channels in different tissues. Different combinations of Kir6.x and SURx yield tissue-specific K_{ATP} channel subtypes with different electrophysiological and pharmacological properties (Fujita & Kurachi, 2000). For example, there is a diversity of K_{ATP} channels encoded by different genes: Kir6.2/SUR1 constitutes K_{ATP} channels in pancreatic â-cells (Inagaki *et al.*, 1996, 1997); Kir6.2/SUR2A in cardiac and skeletal muscles (Inagaki *et al.*, 1996); Kir6.2/SUR2B in non-VSMC and other types of neurons (Liss *et al.*, 1999); Kir6.1/SUR2B in VSMC (Isomoto *et al.*, 1993; Yamada *et al.*, 1997). Functional K_{ATP} channel complex made of Kir6.1/SUR1 has been suggested to be present in glial cells and dentate gyrus granule cells (Skatchkov *et al.*, 2002; Pelletier *et al.*, 2000). Based on the data of pharmacological sensitivity to diazoxide, P-1075, glibenclamide, 5-HD, and HMR-1098, the combination of Kir6.1/SUR1 has been suggested to constitute the molecular makeup of mitochondrial K_{ATP} channels (Liu *et al.*, 2001). This notion is further supported by the identification of both Kir6.1 and SUR1 proteins in mitochondria of P_{12} cells (Tai *et al.*, 2003).

In VSMC, the transcripts of Kir6.1, Kir6.2, SUR2B, and SUR1 have been detected recently and four K_{ATP} channel subunit genes have been cloned from rat mesenteric artery with their full sequences (Cao *et al.*, 2002). Kir6.1 has a ubiquitous

tissue expression, while Kir6.2 has a restricted tissue distribution. Kir6.1 shows ~70% homology in amino acid sequence with Kir6.2. It is possible that VSMC possess multiple types of K_{ATP} channels constructed by Kir6.1 with either SUR1 or SUR2B as the regulatory subunit. This is because Kir6.1 confers the relative ATP insensitivity, which is one of the fingerprints of K_{ATP} channels in VSMC. It is worth noting that a chimeric Kir6.1-Kir6.2 may also occur in native cells because a chimeric Kir6.1-Kir6.2 co-expressed with SUR2 in HEK-293 cells yields functional K_{ATP} channels (Cui *et al.*, 2001). The ability of these cloned subunit genes to form the functional channels has been tested in many heterologous expression systems. Generally speaking, the molecular composition of native K_{ATP} channels in VSMC, including those from mesenteric arteries, is unknown.

1.2.4 Reconstituted K_{ATP} channels with Kir6.1/SUR2B represent vascular K_{NDP} channels

Co-expression of Kir6.1 and SUR2B in HEK-293 cells has been shown to produce K^+ channel currents, which are activated by NDPs like UDP, GDP, and ADP, and blocked by glibenclamide, and is rather insensitive to ATP (Yamada *et al.*, 1997). The electrophysiological and pharmacological properties of Kir6.1/SUR2B channel resemble those of the NDP-dependent (K_{NDP}) channels in VSMC (Zhang & Bolton, 1995; 1996). This notion was supported by the recent observation that Kir6.1 and SUR2B in *in situ* hybridization studies are selectively expressed in the smooth muscle layer of small arterials including mesenteric artery (Li *et al.*, 2003). Thus, the expression of Kir6.1 and SUR2B in these blood vessels suggests that K_{ATP} channels composed of

Kir6.1 and SUR2B are the potential mediators of vascular diameter in accordance with the change of physiological and pathophysiological conditions (Li *et al.*, 2003).

The following experimental evidence supports a functional identity of the native and recombinant channels with respect to their biophysical properties, nucleotide regulation, and pharmacology.

1) Recombinant K_{ATP} channels with Kir6.1/SUR2B have a low unitary conductance of ~35 pS, which is similar to that of vascular K_{NDP} channels (20-40 pS); whereas Kir6.2/SUR2B channels have a considerably higher conductance at 70-80 pS, corresponding to that of classic K_{ATP} channels in VSMC.

2) Co-expressed channels with Kir6.1/SUR2B mimic the nucleotide regulation of vascular K_{NDP} channels. Kir6.1/SUR2B channels exhibit a bell-shaped concentration-dependent regulation by intracellular ATP with maximal activity at ~1 mM (Satoh *et al.*, 1998), similar to that of K_{NDP} channels (Zhang & Bolton, 1996). Spontaneous activity of Kir6.1/SUR2B is not observed on patch excision of the inside-out configuration unless nucleotide diphosphates or triphosphates and Mg^{2+} are present; whereas native K_{ATP} channels in inside-out patches are not activated in ATP-free bath solutions.

3) Kir6.1/SUR2B channels exhibit KCOs and SUR pharmacology that is consistent with that of vascular K_{NDP} channels (Yamada *et al.*, 1997). K_{NDP} and Kir6.1/SUR2B channels are activated by pinacidil and diazoxide. The glibenclamide sensitivity of Kir6.1/SUR2B channels is some fourfold higher than that of Kir6.2/SUR2B.

4) Kir6.1/SUR2B channels mimic the regulation by PKA or PKC etc., which was also demonstrated for native vascular K_{NDP} channels (Hayabuchi *et al.*, 2001).

5) Gene-knockout mice studies provide strong evidence that Kir6.1, but not Kir6.2, is essential for the formation of vascular K_{ATP} channels. Whole-cell K_{ATP}

currents activated by pinacidil and inhibited by glibenclamide were not present in aortic VSMC isolated from Kir6.1 knockout mice, but were identified in myocytes of control and Kir6.2 knockout mice (Suzuki *et al.*, 2001). Moreover, Kir6.1 knockout mice exhibit sudden death as the result of myocardial ischemia caused by abnormal regulation of coronary arteriolar tone and presence of coronary vasospasm (Miki *et al.*, 2002). In contrast, vascular contractility and the hemodynamic profile of Kir6.2 knockout mice were unaltered compared to that of control mice (Suzuki *et al.*, 2001).

1.2.5 K_{ATP} channels in gene-manipulated mice VSMC

Recent studies of Kir6.1 and SUR2 gene-knockout mice have shown that while aortic VSMC of wild-type mice exhibit pinacidil-induced K^+ channel currents that are blocked by glibenclamide, there is no K_{ATP} channel activity in aortic VSMC from both Kir6.1 and SUR2 knockout mice (Miki *et al.*, 2002; Chutkow *et al.*, 2002) and their vasodilating effects by pinacidil are abolished. These findings provided direct evidence that the vasodilation effects of KCOs are mediated by opening of Kir6.1/SUR2 channels (most likely Kir6.1/SUR2B) in VSMC. In Kir6.1 and SUR2 gene-knockout animal models, other physiological functions appeared to be altered. The following features in Kir6.1 gene knockout mice have been known.

a) Blood pressure: KCOs are known to lower blood pressure by relaxing vascular smooth muscles, presumably by opening K_{ATP} channels (Weston & Edwards, 1992). Intravenous injection of pinacidil decreased the mean arterial pressure significantly in control mice but not in Kir6.1 knockout mice, indicating a loss of the vasodilation response to pinacidil in Kir6.1 knockout mice.

b) Tension assay: the vasodilation response of aorta to pinacidil in Kir6.1 knockout mice was also reduced remarkably *in vitro*, as assessed by changes in the isometric tension of aortic rings, compared to that in wild-type mice.

c) K_{ATP} currents: pinacidil elicited significantly K^+ currents that were blocked by glibenclamide in aortic VSMC of wild-type mice, but failed to evoke significant K^+ currents in those of knockout mice, clearly indicating that Kir6.1 is an essential component of K_{ATP} channels in VSMC.

d) Vasospasm: vasospasms were induced in Kir6.1 knockout mice both *in vivo* and *in vitro* by application of methylergometrine, which stimulates serotonergic receptors and directly triggers vasoconstriction of VSMC. Furthermore, the phenotype of Kir6.1 knockout mice resembles that of Prinzmetal angina in humans (Prinzmetal *et al.*, 1959; Maseri, 1987). In similar to Kir6.1 knockout mice, SUR2 knockout mice exhibit significantly elevated resting blood pressures, and the focal narrowing of coronary arteries.

These studies of Kir6.1 and SUR2 knockout mice make it clear that the Kir6.1/SUR2 channel is critical in the regulation of vascular tonus, especially in the coronary arteries. Many signal molecules, including adenosine, NO and other endothelium-derived factors, are thought to participate in the development of coronary vasospasm (Feliciano & Henning, 1999). However, no gene associated with coronary vasospasm has been identified, nor have animal models of coronary vasospasm been available. Accordingly, both Kir6.1 and SUR2 knockout mice are very useful for investigating the molecular mechanisms of pathogenesis of coronary vasospasm. This emphasizes that Kir6.1/SUR2B isoform is an important component of molecular compositions of vascular K_{ATP} channels.

1.3 Modulations of K_{ATP} channels and underlying mechanisms

Under normal circumstances, any particular biological environment, including cellular and extracellular, is predominantly in a reduced state, which is maintained by an array of enzymatic systems and probably under genetic control. The disturbance of the normal reducing environment is called oxidative stress, which results mainly from enhanced production of oxidizing agents and suppressed antioxidant activity. Oxidative stress can cause loss of biological function, the accumulation of toxic oxidation products, and cell death, leading to the occurrence of diseases such as ischemic stroke, cancer, diabetes, and neurodegenerative diseases etc. Oxidative stress is characterized by the presence of unusually high concentration of toxic reactive species, principally consisting of reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive nitrogen oxygen species (RNOS), reactive sulfur species (RSS) and unbound adventitious metal ions (reactive iron species), etc. (Betteridge, 2000; Giles *et al.*, 2001). These species are highly oxidizing, readily destroying redox-sensitive proteins and enzymes as well as attacking membranes and DNA. In contrast, molecules containing sulfur are generally considered to act as antioxidants. Thiols serve as cellular redox buffers, reducing ROS/RNS and so maintaining the overall redox state of the cell. Being one of the most important membrane proteins that are sensitive to metabolic products, the K_{ATP} channel is a target for these reactive species. The modulation of K_{ATP} channels by thiol, ROS, RNS, and H_2S will be discussed in the following chapters

1.3.1 Modulation of K_{ATP} channels by thiol redox

1.3.1.1 Free thiols and thiol reducing buffer

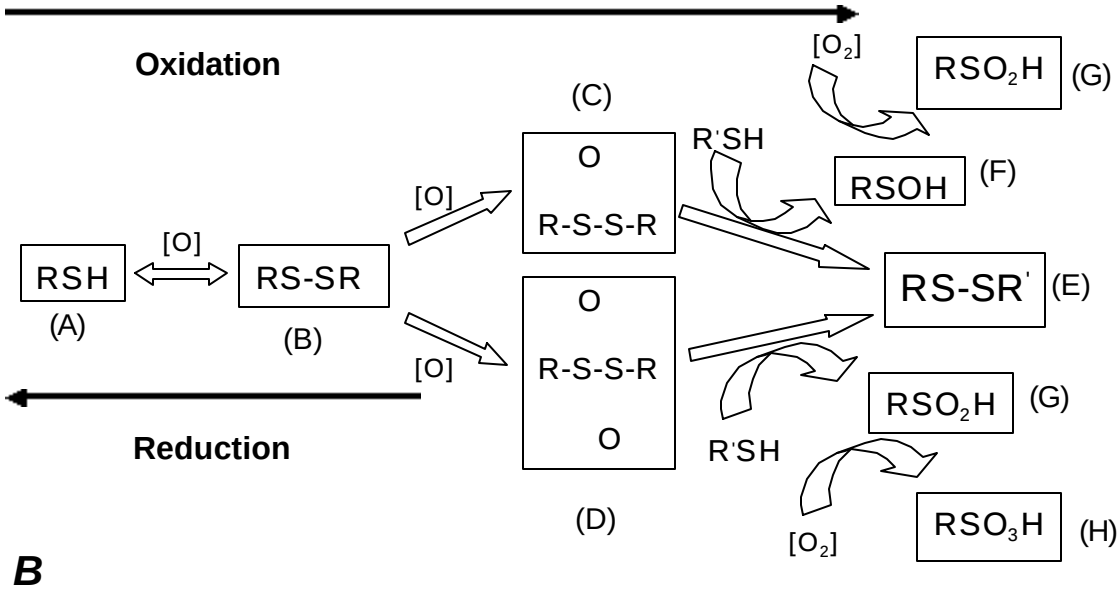
To counteract the effects of oxidative stress, cells have developed two important defense mechanisms: a thiol reducing buffer (GSH and thioredoxin), and enzymatic systems (SOD, catalase, and glutathione peroxidase). Thiol groups are those which contain functional –SH groups within conserved cysteinyl residues. There are many naturally occurring thiols. The simplest thiol is H_2S gas. The most abundant biologically occurring thiol is the amino acid cysteine, along with its disulfide cystine. The most important thiol is the cellular redox buffer GSH, present within cells at a millimolar concentration. Thiols also exist in different cysteine-containing compounds such as amino acids (cysteine, cystathionine, taurine, homocysteine), peptides (GSH, Co-enzyme A), and proteins (thioredoxin, glutaredoxin, albumin, metallothionein, glutathione peroxidase, peroxiredoxin, redox factor-1, heat shock protein, etc.). By virtue of their ability to be reversibly oxidized, thiols are recognized as key components involved in the maintenance of redox balance, at which overall reducing conditions prevail within cells. GSH acts as a first line of defence to detoxify different reactive species like ROS and RNS. GSH peroxidase catalyzes the reduction of ROS and RNS via the oxidation of GSH thiol to GSSG disulfide. Additionally, thiol groups located on various molecules act as redox sensitive switches, thereby providing a common trigger for a variety of ROS- or RNS-mediated signaling events.

1.3.1.2 The oxidation of thiols in cellular redox signaling

Within biological systems, thiols undergo reversible and irreversible oxidations when exposed to oxidative stress. The former is a weak redox process via the thiol-disulfide exchange; whereas the latter is a strong redox process, which is often catalysed by transition metals like iron and involves free radicals as intermediates. Reversible oxidation of thiols (cysteine) has been postulated to work as an important cellular redox sensor in some proteins (Finkel, 2000). Physiologically, the disulfide formation may be the most likely consequence of cysteine oxidation. Disulfides can be easily reduced back to thiols using GSH *in vivo* or DTT *in vitro*. Proteins containing –SH/S–S groups can interact with GSH in a thiol-disulfide exchange, which is often utilized for the reduction of both intra- and inter-molecular disulfides in proteins. In essence, thiol-disulfide exchange can be described as a redox process because the oxidation state of the sulfur atoms changes in the direction of greater electron deficiency in the disulfide. At physiological condition (pH 7 and 25ºC), the equilibrium constants are usually near unity. Therefore, a large excess of thiol must be used to reduce a disulfide and *vice versa*. Thiols and their disulfides are reversibly linked, via specific enzymes, to the oxidation and reduction of NAD(P) and NAD(P)H systems (Moran *et al.*, 2001).

On the other hand, when exposed to strong oxidizing agents, thiols are converted to disulfides. The disulfides are irreversibly oxidized to disulfide-S-oxides, and finally to sulfenic (R-SOH), sulfinic (R-SO_2H), and sulfonic (R-SO_3H) acids (Fig. 3A). Disulfide-S-oxides, including monoxide and dioxide, are important RSS, which oxidize thiols to form mixed disulfides in the process of generating sulfenic or sulfinic acids (Fig. 3A). Disulfide-S-oxides are formed from GSH thiols and disulfides in the presence of H_2O_2 at low or neutral pH; whereas at higher pH values, sulfinic and sulfonic acids are formed

A



B

a. Reversibly oxidized forms of protein thiols (Pr-SH)

1 electron oxidations

(1) Pr-SH + R· ⇌ RH + Pr-S· (thiyl radical)

2 electron oxidation

(2) Pr-S⁻ + RSSR ⇌ RS⁻ + Pr-S-SR (disulfide)

(3) Pr-SH + N_2O_3 ⇌ HNO_2 + Pr-SNO (nitrosothiol)

(4) Pr-SH + H_2O_2 ⇌ H_2O + Pr-SOH (sulfenic acid)

(5) Pr-SH + H_2O_2 + GSH ⇌ $2H_2O$ + Pr-SSG (S-thiolated)

(6) Pr-SH + R· + GSH + O_2 ⇌ $2H_2O$ + RH + $O_2^{\cdot -}$ + Pr-SSG (S-thiolated)

b. Irreversibly oxidized forms of protein thiols (Pr-SH)

2 electron oxidations

(7) Pr-SOH + O_2 ⇌ Pr-SO_2H (sulfinic acid)

(8) Pr-SO_2H + O_2 ⇌ Pr-SO_3H (sulfonic acid)

Fig. 3: Reactions producing oxidized forms of thiols. A. Oxidative formation of disulfide-S-oxide and subsequent mixed disulfide. Disulfide (B) is formed from thiols (A) under oxidative stress [O]. The disulfide bond is also reduced to thiol group. Steps that are reversible are also shown ( ). Further oxidation of disulfide yields either disulfide-S-monoxide (C) or disulfide-S-dioxide (D). Activation of disulfide via sulfur oxidation renders the bond more labile and promotes the reaction with a reduced thiol to form the mixed disulfide (E) and sulfenic (F), sulfinic (G), sulfonic (H) acids. **B.** Protein thiols (Pr-SH) are oxidized reversibly and irreversibly with 1 or 2 electrons to form thiyl radical (1), the disulfide (2), sulfenic (4), sulfinic (7), sulfonic acids (8), S-thiolated (5 & 6), and nitrosothiol (3).

by alkaline dismutation (Giles *et al.*, 2001). The rapid reaction of disulfide-S-oxides with protein thiols to form mixed disulfides may unveil the mechanism of protein thiolation, which is not thought to be mediated enzymatically, rather it occurs in response to cellular oxidizing species. Thus, thiols of redox proteins may also be directly modified by ROS to form oxidized species such as S-thiolated (RSSG), disulfide (RS-SR), and sulfe(i,o)nic acid (RSOxH). In addition, thiols of redox proteins may also be oxidized by RNS to form nitrosated species like nitrosothiol (RSNOx) (Fig. 3B). The S-thiolation and N-nitrosylation induced by S-thiolated and nitrosated species, respectively, are proposed to occur in a wide range of diverse signal transduction pathways, possibly contributing to the molecular mechanisms of the actions of H_2S and NO on K_{ATP} channels.

Therefore, oxidation of cellular thiols not only inhibits the activity of redox proteins and enzymes, but also consumes GSH, and hence tilts the cellular redox balance towards oxidative stress. The change in redox state is sensed by thiol-containing proteins via a thiol modification. There is growing evidence that redox sensing proteins play roles in mediating cellular responses to oxidative stress, such as the activation of certain nuclear transcription factors (NF-*ê*B) and the determination of cellular fate by apoptosis or necrosis. K_{ATP} channel proteins contain multiple cysteine residues. Whether the reducing agent H_2S and the oxidizing agent chloramine T (CLT) modulate K_{ATP} channel activity and how they work should be investigated systematically.

1.3.1.3 Modulation of K_{ATP} channels by thiol oxidizing and reducing agents

K_{ATP} channel protein contains critical thiol groups, which may sense changes in the metabolism and in the redox potential of cells (Islam *et al.*, 1993; Tricarico *et al.*,

1994, 2000; Linde *et al.*, 1997). ROS and RNS are very strong oxidizing agents. They may switch the functional thiol groups from the reduced to the oxidized, altering the activity of K_{ATP} channels (Stadtman, 1992; Bernardi *et al.*, 1992; Lee *et al.*, 1994; Coetzee *et al.*, 1995; Weik & Neumcke, 1989; Trapp *et al.*, 1998). In addition, thiols can be oxidized by a series of oxidizing agents and their corresponding disulfides reduced by reducing agents. Specific –SH group oxidizing agents are composed of hydrophilic 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB), lipophilic 2, 2'-dithio-bis(5-nitropyridine) (DTBNP) and 4, 4'-dithiodipyridine (4-PDS), membrane-impermeant organic mercurial compounds like [(O-carboxyphenyl) thiol] ethyl-mercury sodium salt (thimerosal), p-chloromercuri-phenylsulfonic acid (p-CMPS), p-hydroxymercuri-phenyl-sulfonic acid (p-HMPS), and 4-hydroxy-mercuribenzoic acid (PMB) etc. DTNB and 4-PDS are known to oxidize free –SH groups through a thiol-disulfide exchange mechanism. In contrast, thimerosal and organic mercurial compounds bind to –SH groups via an S-Hg interaction (Cai & Sauve, 1997). Other thiol oxidizing agents include N-ethylmaleimide (NEM), H_2O_2, CLT, and GSSG. NEM is often used as a sulfhydryl alkylation compound. CLT oxidized not only –SH group of cysteine residues, but also thioether group of methionine residue of protein (Shechter *et al.*, 1975). On the other hand, the disulfide reducing agents include dithiothreitol (DTT), GSH, and H_2S etc. (Wei & Neumcke, 1989; Caputo *et al.*, 1994; Trapp *et al.*, 1998). Nearly all –SH oxidizers, such as DTNB, DTBNP, thimerosal, NEM, CLT, p-CMPS etc., inhibited K_{ATP} channel activity in different tissues and cells (Islam *et al.*, 1993; Coetzee *et al.*, 1995; Han *et al.*, 1996) except H_2O_2 activated K_{ATP} channels (Chiandussi *et al.*, 2002); whereas the reducing agents like DTT and GSH were not found to alter K_{ATP} channel activity when used alone. However, they often rapidly reversed the inhibition of K_{ATP} channel activity

by certain –SH oxidizers like DTBNP, thimerosal, and pCMPS, etc. (Islam *et al.*, 1993; Coetzee *et al.*, 1995; Han *et al.*, 1996; Trapp *et al.*, 1998). Whether or not these –SH group oxidizers (CLT) and reducers (H_2S) affect K_{ATP} channels in VSMC has not been determined. How the reducing agent H_2S acts on K_{ATP} channels in VSMC is unknown.

1.3.2 Modulation of K_{ATP} channels by ROS

1.3.2.1 Classification and function of ROS

Cells constantly generate reactive oxygen species (ROS) during aerobic metabolism. ROS are produced as by-products of oxidative metabolism, in which energy activation and electron reduction are involved. ROS includes free radicals such as the superoxide anion (O_2^-), hydroxyl radicals (·OH), and the nonradical hydrogen peroxide (H_2O_2). They are particularly transient species due to their high chemical reactivity and can react with DNA, proteins, enzymes, and ion channels, etc. ROS production is enhanced severely in several disease states such as hypertension, diabetes mellitus and atherosclerotic coronary artery. However, ROS production at the sub-toxic level may also serve to provide molecules for biological signaling. For example, sub-lethal hyperoxia can mediate protection upon re-exposure by mechanisms involving the up-regulation of protective antioxidant enzyme systems (Fleming *et al.*, 1991; Das *et al.*, 1999). This in part involves signaling by ROS production during hyperoxia. Thus, ROS-derived oxidative damage probably represents an extreme and toxic event although ROS molecules may have useful signal functions.

1.3.2.2 Generation of ROS

The mitochondria are a major site of generation of free radicals (Wolin, 1996). Under physiological conditions, 1-2% of the electrons carried by the electron transport chain can leak out of the pathway and pass directly to oxygen, generating O_2^-. Complex I (NADH-ubiquinone oxidoreductase) and Complex III (ubiquinol-cytochrome *c* oxidoreductase) are the two sites where O_2^- is produced (Beyer, 1992). Other sources of O_2^- include various enzymes such as cytochrome *P450* in the endoplasmic reticulum, lipoxygenases, cyclooxgenases, xanthine oxidase and NADPH oxidase (Wolin, 1996). Xanthine oxidase (XO) metabolizes hypoxanthine (HX), xanthine (X), and NADH to form O_2^- and H_2O_2. Ischemia and hypoxia are conditions that promote the accumulation of these substrates for ROS production and the increase in xanthine oxidase activity. O_2^- is produced with a 1-electron reduction of molecular oxygen by various oxidases (*Equation 1:* O_2 + electron O_2^-). O_2^- is a negatively charged free radical that undergoes rather selective chemical reactions with the components of biological systems. Although O_2^- reacts with itself with a rate constant of 8×10^4 $M^{-1}\cdot s^{-1}$ to form H_2O_2 and O_2 (*Equation 2:* $O_2^- + O_2^- + 2H^+$ $H_2O_2 + O_2$), superoxide dismutase (SOD) functions to accelerate the removal of O_2^- as a result of its rate constant of 2×10^9 $M^{-1}\cdot s^{-1}$ for the reaction with O_2^-. One of the most important roles of SOD is the prevention of the reaction of O_2^- with NO (*Equation 3:* O_2^- + NO $OONO^-$) (Fig. 4). H_2O_2 is a relatively stable species. It is either derived from O_2^- through *Equation 2*, or it is directly produced by certain oxidases through a 2-electron reduction of O_2. The reaction of H_2O_2 with ferrous ion results in the formation of ·OH (*Equation 4:* $Fe^{2+} + H_2O_2$ $Fe^{3+} + \cdot OH + OH^-$) (Wolin, 2000). When X or HX is oxidized by XO in the presence of oxygen, an electron from the reaction of X/HX with XO is transferred to oxygen to form O_2^-. The

dismutation of O_2^- generates H_2O_2 via cytosolic or mitochondrial SOD. Further oxidation of H_2O_2 by a transition metal such as ferrous iron leads to highly potent ·OH via a Fenton-type reaction (*Equation 4*) and the metal catalyzed Haber-Weiss reaction (*Equation 5:* $O_2^- + H_2O_2 \rightarrow O_2 + \cdot OH + OH^-$) (Graf *et al.*, 1984; Yu, 1994).

1.3.2.3 Scavengers of ROS and antioxidant system

The cell possesses numerous antioxidants to buffer the generation of oxidizing agents with potential damage and prevent oxidative damage directly by intercepting ROS before they can damage intracellular targets. The antioxidant system consists of SOD, glutathione peroxidase (GPx), catalase, serum aminooxidase, and thirodoxin reductase. Four classes of SOD (Mn-SOD, Cu, Zn-SOD, Ni-SOD and extracellular SOD) have been identified to date. All four SOD enzymes destroy O_2^- by converting it to H_2O_2 as *Equation 2*. Other scavengers for O_2^- include cell membrane-permeable tetramethylpiperidine-N-oxyl (TEMPO) and 4, 5-dihydroxy-1, 3-benzene disulfonic acid (Tiron). H_2O_2 is one of the major ROS in the cell. The primary defence mechanisms against H_2O_2 are catalase (Michiels *et al.*, 1994) and GPx through the glutathione (GSH) redox cycle (Reed, 1990). Catalase is one of the most efficient enzymes known (Lledias *et al.*, 1998). It is present only in the peroxisome fraction whereas the GSH redox cycle exists in the cytosol and mitochondria. Catalase reacts with H_2O_2 to form water and molecular oxygen ($2H_2O_2 \rightarrow 2H_2O + O_2$). ·OH is sourced from H_2O_2 via *Equation 4 & 5*. The scavengers for · OH are dimethylthiourea and mannitol. Serum aminooxidase reacts with · OH to form water in the presence of electron donor ($\cdot OH + H^+ \rightarrow H_2O$).

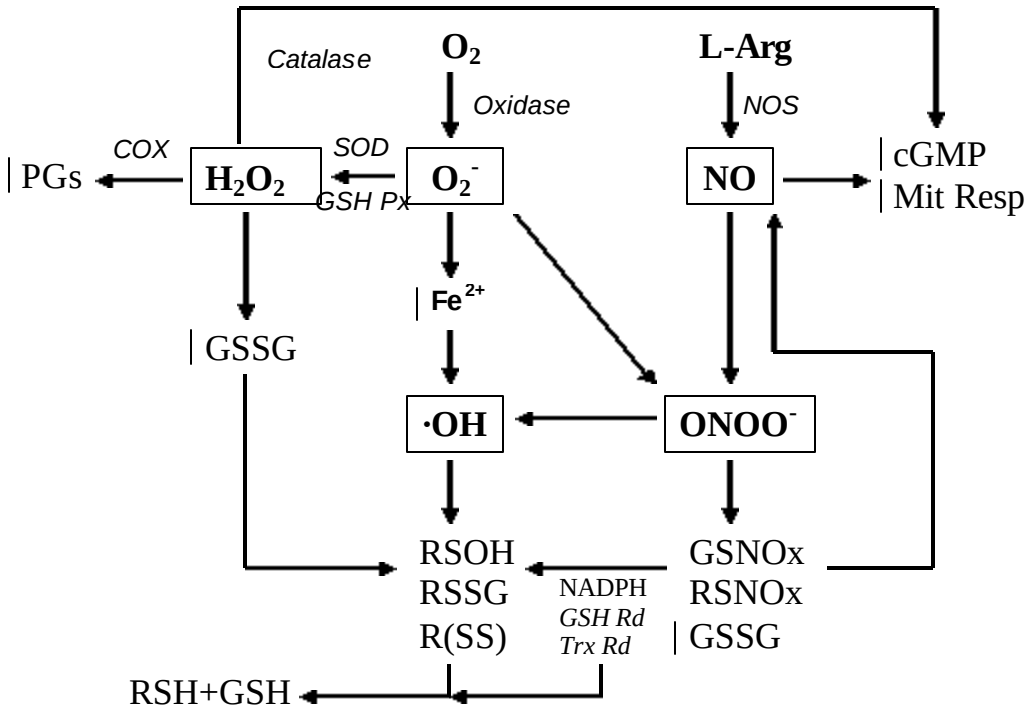


Fig. 4: Origins of oxidant species potentially involved in vascular signalling mechanisms. Some of the direct interactions of species derived from the formation of O_2^- and NO with signalling systems that are often active under basal physiological conditions are included. O_2^- inactivates NO, prevents sGC activation, and generates $OONO^-$. H_2O_2 stimulates sGC via catalase, forms GSSG by GSH peroxidase (Px), and generates PGs via COX. ·OH oxidizes thiols and generates lipid oxidation products. NO stimulates sGC and inhibits reversibly mitochondrial respiration (Mit Resp) via cytochrome oxidase. $OONO^-$ formed by O_2^- and NO generates nitrothiols via oxidizing thiols. Oxidases include NADH/NADPH oxidase, xanthine oxidase etc. Rd indicates reductase; Trx, thioredoxin.

The GSH system is probably the most important cellular defence mechanism that exists in the cell. The tripeptide GSH (ã-Glu-Cys-Gly) not only acts as an ROS scavenger but also functions in the regulation of the intracellular redox state. The system consists of GSH, GPx and glutathione reductase. GPx catalyses the reduction of H_2O_2 and other peroxidases and converts GSH to its oxidized disulfide form (GSSG) (ROOH + 2GSH ROH + GSSG + H_2O). GSSG is then reduced back to GSH by glutathione reductase (GSSG + RSH GSH + RSSG). The ability of the cell to regenerate GSH either by reduction of GSSG or new synthesis of GSH is an important factor in the efficiency of that cell in managing oxidative stress (Fig. 4). Under normal conditions, more than 95% of the GSH in a cell is reduced and so the intracellular environment is usually highly reducing. Each cell is equipped with an extensive antioxidant defense system to combat excessive production of ROS and prevent the occurrence of oxidative stress (Carmody *et al.*, 1999).

1.3.2.4 Modulation of K_{ATP} channels by ROS

The modulation of K^+ channel activity by cellular oxidative stress has emerged as a significant determinant of vascular tone (Liu & Gutterman, 2002; Sobey *et al.*, 1997, 2001). Different kinds of ROS were reported to modify various types of K^+ channels in vascular tissues (Liu & Gutterman, 2002; Pomposiello *et al.*, 1999). However, the modulation of K_{ATP} channels by diverse ROS is unclear. H_2O_2 elicited a glibenclamide-sensitive dose-dependent dilation of cat cerebral arterioles and rat gracilis skeletal muscle arterioles (Wei *et al.*, 1996; Iida & Katusic, 2000; Cseko *et al.*, 2004). The dilation of cerebral and coronary arteries by $OONO^-$ is blocked by glibenclamide. This suggests a role of K_{ATP} channels (Liu *et al.*, 1994, 2002; Wei *et al.*, 1996, 1998).

Application of HX/XO together with $FeCl_3$ to pial artery *in vivo* led to a significant reduction in the vasodilatatory responses to K_{ATP} channel agonists (cromakalim and calcitonin gene-related peptide) (Armstead, 1999; Bari *et al.*, 1996). However, one cannot conclude that O_2^- inhibits K_{ATP} channel in these VSMCs, because changes in diameter of pial artery *in vivo* are under influences of many vasoactive substances that act by diverse mechanisms. Furthermore, direct effect of HX/XO on the basal diameter of pial artery was not examined in the above study. Although HX/XO is widely used as the free radical generating system, the direct electrophysiological evidence for the effects of HX/XO on K_{ATP} channel activity is lacking in VSMC. Whether HX or X/XO-generating O_2^- directly alters K_{ATP} channel activity has never been reported.

1.3.3 Modulation of K_{ATP} channels by NO and NO-derived RNS

1.3.3.1 Production and function of NO and RNS

NO is either delivered by NO donors like SNP and SNAP or endogenously produced from L-arginine by the NO synthase (NOS) (Southam & Garthwaite, 1991). Endogenous NO synthesis occurs in the cytoplasm by a two-step oxidation of L-arginine to L-citrulline by NOS via formation of N^{ω}-hydroxyl-L-arginine as an intermediate. NOS is a family of NADPH-dependent enzymes and exists in four major isoforms. While neuronal NOS (nNOS) and endothelial NOS (eNOS) are Ca^{2+}/calmodulin-dependent and constitutively expressed in a wide variety of cells, inducible NOS (iNOS) is Ca^{2+} independent and is expressed in cells of the immune system and other cells in response to various stimuli (Nathan, 1997). The activation of eNOS or nNOS usually produces small amounts of NO, while the induction of iNOS generates large amounts of

NO (Kroncke *et al.*, 1995). NADPH oxidase catalyses the direct reduction of molecular oxygen to O_2^-. NOS also produced O_2^- under conditions of reduced substrate (L-arginine) or decreased tetrahydrobipterin, a cofactor for NOS (Heinzel *et al.*, 1992). The most recently discovered mitochondrial NOS (mtNOS) is present exclusively in the mitochondria (Bates *et al.*, 1995, 1996; Tatoyan & Giulivi, 1998). Co-stimulation of O_2^- production and mtNOS can result in the formation of high concentrations of highly reactive and damaging $OONO^-$ (Packer *et al.*, 1996; Bringold *et al.*, 2000).

Reactive nitrogen species (RNS) are generated by the interaction of O_2 and ROS with NO and possess additional oxidant signalling properties. The important RNS are mainly $ONOO^-$, nitrogen dioxide (NO_2) and N_2O_3. Because O_2^- reacts with NO with a rate constant of 7×10^9 $M^{-1}\cdot s^{-1}$, which is over 3 times the rate of its reaction with SOD, when NO concentration increases into the range of the tissue levels of SOD, NO competes with SOD for scavenging of O_2^- by forming $OONO^-$ (Wolin, 1996). Under conditions such as ischemia, an excessive amount of O_2^- can also react with NO and generate $OONO^-$, which can interact with a variety of molecules (Beckman & Koppenol, 1996; Pryor & Squadrito, 1995). Although $OONO^-$ has been attributed to NO-induced cell injury (Lipton *et al.*, 1993), it could also have a potentially important role in signal transduction mechanisms (Stamler, 1994; Wolin, 1996). For example, thiol oxidation has been suggested to be an important mechanism through which $OONO^-$ can directly modulate K^+ channel activity (Bolotina *et al.*, 1994; Busch *et al.*, 1995; Lu & Wang, 1998). NO_2 appears to be produced in significant amounts from $OONO^-$ because significant amounts of NO_2 may be formed from the reaction of O_2 with NO levels in the high nanomolar range or greater (Liu *et al.*, 1998). This is due to the greater solubility of NO and O_2 in hydrophobic environments. $OONO^-$ can decompose to yield further

oxidants with the chemical reactivity of $\cdot NO_2$, $\cdot OH$ and NO_2^+. N_2O_3 is formed through the binding of NO with NO_2.

1.3.3.2 Action mechanisms of NO and $OONO^-$

NO exists in different chemical forms (NO^-, $NO\cdot$ and NO^+). Non-radical NO is poorly reactive with most molecules within cells, but, as a free radical, it can react extremely rapidly with other free radicals such as O_2^-, amino acid radicals, and certain transition metal ions. Thus NO radical has a wide-ranging degree of chemical reactivity and functions in a variety of different biological roles (Stamler *et al.*, 1992) such as the regulation of the cardiovascular system, smooth muscle relaxation, neurotransmission, coagulation and immune regulation. NO has been reported to have protective effects against cardiovascular diseases such as angina, hypertension, diabetes, etc. Three major mechanisms underlie NO actions.

1) The cGMP-dependent mechanism, NO can stimulate cGMP production through the activation of soluble guanylyl cyclase (sGC), which catalyzes the conversion of GTP into the second-messenger molecule cGMP. cGMP is able to modulate, either directly or by activating kinases, the activity of numerous cellular proteins including K^+ channels. Thus, the influence of NO on K^+ channel activity may be secondary to the activation of sGC (Robertson *et al.*, 1993; Archer *et al.*, 1994).

2) The cGMP-independent mechanisms --- *S*-nitrosylation of proteins. *S*-nitrosylation involves the transfer of a nitric group to cysteine sulfydryls, leading to the formation of a nitrosothiol (RSNO). While the movement and activity of NO is often restricted due to its very short half-life, nitrosothiols in comparison are very stable compounds and function as tissue storage forms and donors of NO. For example, NO

may directly affect K_{Ca} channel activity by interacting with thiol groups of channel protein because the thiol-depleting agent NEM prevented the NO-induced activation of K_{Ca} channels. Accordingly, it is likely that NO nitrosates the thiols of the channel protein and this confers the activation of the K^+ channel. NO was also reported to directly activate K_{Ca} channels in VSMC (Bolotina *et al.*, 1994; Mistry & Garland, 1998).

3) The formation of $ONOO^-$ from NO reaction with O_2^- is an important physiological pathway to inactivate NO biologically. $OONO^-$ shares some properties of NO in that it can diffuse freely intra- and inter-cellularly and also acts as a powerful oxidant. The most potent effects of $OONO^-$ appear to be thiol modifications that either affect the function of signaling systems or result in the production of tissue-derived donors of NO (Radi *et al.*, 1991). $OONO^-$ readily interacts with GSH and other thiols in tissues to cause thiol oxidation or the formation of nitrated ($RSNO_2$) or nitrosated (RSNO) thiols. NO disrupts mitochondrial function by reversibly inactivating cytochrome *c* oxidase and terminal electron acceptor in the respiratory chain, thus stimulating O_2^- generation, $OONO^-$ production, and ATP depletion (Fig. 4) (Cleeter *et al.*, 1994; Cassina & Radi, 1996). Furthermore, in the presence of NO donor, X/XO may form powerful $OONO^-$, which complexes the effects of single O_2^- or NO on K^+ channels. It is reported that L-arginine was converted into NO and O_2^- through hydroxylamine (HA) intermediate by catalase in the presence of H_2O_2 (Pou *et al.*, 1991). Whether HA-generated NO and O_2^- can form $OONO^-$ has never been known.

1.3.3.3 Modulation of K_{ATP} channels by NO

Although NO has been suggested to regulate K_{Ca} channels in VSMC either directly or indirectly, the role of NO in the regulation of K_{ATP} channel function in

different vascular beds is controversial (Liu *et al.*, 2002; Wu *et al.*, 2002; Bolotina *et al.*, 1994; Mistry & Garland, 1998). At the tissue level, the dilator responses to SNP in porcine pial artery were significantly diminished in the presence of K_{ATP} channel antagonist, glibenclamide. This suggests a definitive role for K_{ATP} channels in mediating NO induced vasodilatation (Armstead, 1996, 1997). In contrast, another study has shown that the dilator responses to SNP in rat aortic rings remained unaffected in the presence of glibenclamide (Huang, 1998). At the single VSMC level, both SNP and 8-Br-cGMP failed to evoke any appreciable increases in whole-cell K_{ATP} currents. These data suggest that NO-sGC-cGMP-PKG mediated vasodilatation may not be linked to K_{ATP} channel activation (Wellman *et al.*, 1998; Quayle *et al.*, 1994). However, others have shown that L-arginine, a precursor that enhances endogenous NO production, activated unitary K_{ATP} currents in cell-attached patches via the activation of sGC in cultured VSMC isolated from porcine coronary artery (Kubo *et al.*, 1994; Miyoshi *et al.*, 1994). In addition, the involvement of K_{ATP} channel activation in NO-induced hyperpolarization of smooth muscle is also controversial. SNP activated increases in glibenclamide sensitive membrane hyperpolarization in rabbit mesenteric artery was accompanied by activation of PKG (Lincoln *et al.*, 1994; Murphy & Bryden, 1995). Others have reported that addition of agonists that mimic NO, to rabbit cerebral and canine coronary arteries, failed to evoke hyperpolarization (Komori *et al.*, 1988; Tare *et al.*, 1990; Himmel *et al.*, 1993).

1.3.3.4 Modulation of K_{ATP} channels by $OONO^-$

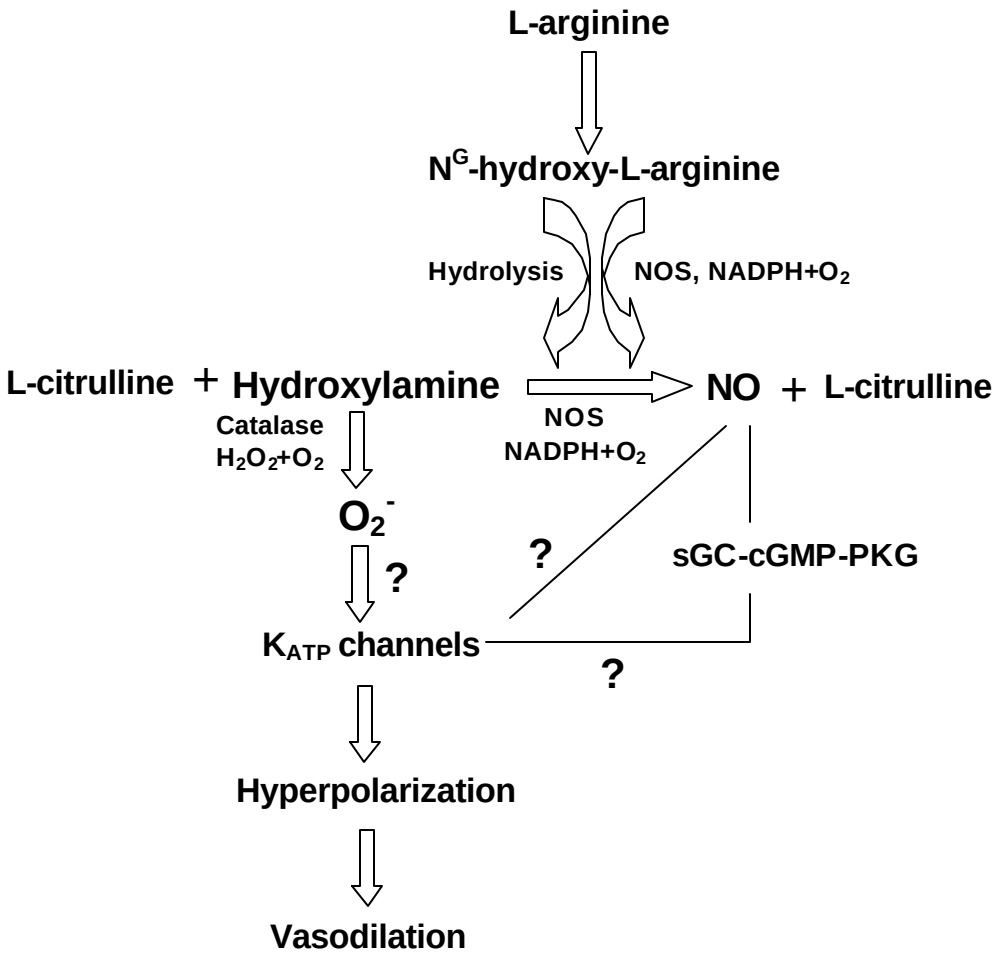
$OONO^-$ is a powerful oxidant and cytotoxic agent that can damage DNA, membrane lipids and mitochondria. It has been shown to modify proteins at methionine,

cysteine, tyrosine, and tryptophan residues (Ischiropoulos and al-Mehdi, 1995; Beckman & Koppenol, 1996; Souza *et al.*, 1999; Ischiropoulos, 2003). K_{ATP} channels are important membrane proteins and they are susceptible to oxidation by $OONO^-$. At the whole-animal level, in pentobarbital-anaesthetized rats, systemic administration of $OONO^-$ produces pronounced hypotensive response due to vasodilatation attained in different vascular beds. This leads to a dose-dependent decrease in hindquarter and mesenteric artery resistance that leads to a significant decrease in mean arterial blood pressure (Kooy & Lewis, 1996; Kooy *et al.*, 1996). Despite this overwhelming evidence, the cellular mechanisms that may govern the profound vasodilator and hypotensive effects evoked by $OONO^-$ have not been fully established. However, the repetitive administration of $OONO^-$ to elucidate its role could result in the rapid development of tachyphylaxis, which may be due to a direct modification of K_{ATP} channels by $OONO^-$ in VSMC. At the tissue level, $OONO^-$ exhibits NO-like biological activity *in vitro* and elicits vasodilation in several vascular beds including coronary, renal, mesenteric and and cerebral arteries (Benkusky *et al.*, 1998; Liu *et al.*, 1994; Wei *et al.*, 1996, 1998). $OONO^-$ induced dose-dependent dilatation of cat cerebral artery and this was inhibited by glibenclamide, an inhibitor of K_{ATP} channels (Wei *et al.*, 1996). In addition, $OONO^-$ may also directly hyperpolarize VSMC via the activation of K_{ATP} channels at physiological pH ranges despite its short half live and suscptibility to rapid degradation (Wei *et al.*, 1996; Pan *et al.*, 2004). In cerebral and coronary arteries, dilatation evoked by $OONO^-$ was blocked by glibenclamide. Addition of $OONO^-$ reduced prostacyclin evoked vasodilatation through a mechanism involving K_{ATP} channels in renal and mesenteric arteries (Wei *et al.*, 1996; Kooy & Lewis, 1996; Kooy *et al.*, 1996).

NO is known to interact with O_2^- to form $OONO^-$ (Pryor & Squadrito, 1995). The NO donor, S-nitroso-N-acetyl-penicillamine (SNAP) at 400 µM evoked membrane hyperpolarization was attenuated by a K_{ATP} channel blocker, glibenclamide, and a scavenger of O_2^-, tiron, suggesting a role for $OONO^-$ and O_2^-. These observations support a role for K_{ATP} channels and O_2^- in mediating NO-evoked hyperpolarization (Zhao *et al.*, 2000). The potential of NO as a profound vasodilator agonist is due to its ability to interact with O_2^- to form $OONO^-$. Moreover, $OONO^-$ has been shown to decrease both vascular resistance and blood pressure in a concentration dependent manner (Kooy & Lewis, 1996; Kooy *et al.*, 1996). However, the evidence in support of this finding at the single cell level with the electrophysiological data that $OONO^-$ directly activates K_{ATP} channels and hyperpolarizes the cell membrane is lacking. Whether $OONO^-$ formation underlies NO-induced activation of K_{ATP} channels and resultant hyperpolarization is intriguing.

1.3.3.5 Modulation of K_{ATP} channels by hydroxylamine

The biosynthesis of NO from its endogernous precursor, L-arginine, is accompanied by the generation of hydroxylamine (HA). HA in turn can be utilized in the regeneration of NO (Ohta *et al.*, 1997; Klink *et al.*, 2001). In addition, it is possible that HA could promote generation of O_2^- via a catalase dependent mechanism that could lead to the activation of K_{ATP} channels (Craven *et al.*, 1979) (Fig. 5). The vasodilator effect of HA in different vascular bed preparations and isolated vascular tissues have been attributed to HA-derived generation of NO (Taira *et al.*, 1997; DeMaster *et al.*, 1989; Moore *et al.*, 1989). However, the exact molecular mechanism underlying HA induced vasodilator effects are unclear in VSMC and how the catalase-derived O_2^- contribute to



Eq-1. $E[Fe^{III}] + \mathbf{H_2O_2} \longrightarrow \mathbf{E[Fe^{V}=O]} + H_2O$

Eq-2. $E[Fe^{V}=O] + \mathbf{HA} \longrightarrow H_2O + E[Fe^{III}HNO]$

Eq-3. $E[Fe^{III}HNO] \longrightarrow E[Fe^{II}NO] + H^+$

Eq-4. $E[Fe^{II}NO] \longrightarrow E[Fe^{II}] + \mathbf{NO}$

Eq-5. $E[Fe^{II}] + \mathbf{O_2} \longrightarrow E[Fe^{III}] + \mathbf{O_2^-}$

Fig. 5: Proposed pathway for the conversion of L-arginine to NO and O_2^- through a hydroxylamine intermediate. Hydroxylamine (HA) is oxidized in a three-electron oxidation to NO and O_2^- by catalase. This is achieved through the following series of reaction (Eq-1-5). Catalase compound I ($E[Fe^{V}=O]$) in Eq-2 oxidizes HA to NO through a ferricatalase-nitroxyl intermediate ($E[Fe^{III}HNO]$). Ferrocatalase ($E[Fe^{II}]$) is converted back to ferricatalase ($E[Fe^{III}]$) by molecular oxygen with the generation of O_2^-, which elicits K_{ATP} channel activation and membrane hyperpolarization of VSMCs, causing vessel dilation.

K_{ATP} channel activation has not been evaluated. HA is known to activate pancreatic β-cell K_{ATP} channels and inhibit insulin release from perfused islets (Antoine *et al.*, 1996). HA-induced relaxation of rat aortic rings was inhibited by different K^+ channel blockers except glibenclamide, eliminating the possibility of a role for K_{ATP} channel (Huang, 1998). However, the action of HA on K_{ATP} channels in VSMC is unknown.

On the other hand, HA is a potent inhibitor of H_2S-generating enzyme, cystathionine β-synthase (CBS) and HA-generated NO enhances the activity of H_2S-generating enzyme cystathionine γ-lyase (CSE) (Fig. 6). Therefore, HA may affect the endogenous H_2S production, and then alter the cardiovascular functions of H_2S, including K_{ATP} channel activation, smooth muscle relaxation, vessel dilatation, and hypotension.

1.3.4 Modulation of K_{ATP} channels by H_2S

1.3.4.1 Physical and chemical properties of H_2S

H_2S is a colorless gas with a strong odor of rotten eggs. The detectable level of this gas by the human nose is at a concentration 400-fold lower than the toxic level (Wang, 2002). H_2S gas is readily oxidized to form sulfur dioxide, sulfates, or elemental sulfur ($2H_2S + 3O_2 \leftrightarrow 2H_2O + SO_2$; $2H_2S + O_2 \leftrightarrow 2H_2O + 2S$). In aqueous solution, H_2S can be hydrolyzed to hydrosulfide ions (HS^-) and sulfide ions (S^{2-}) in the following sequential reactions: ($H_2S \leftrightarrow HS^- + H^+ \leftrightarrow S^{2-} + 2H^+$). This reaction is dependent on the pH of the solution and pKa of the gas. At the physiological pH (7.4), approximately one-

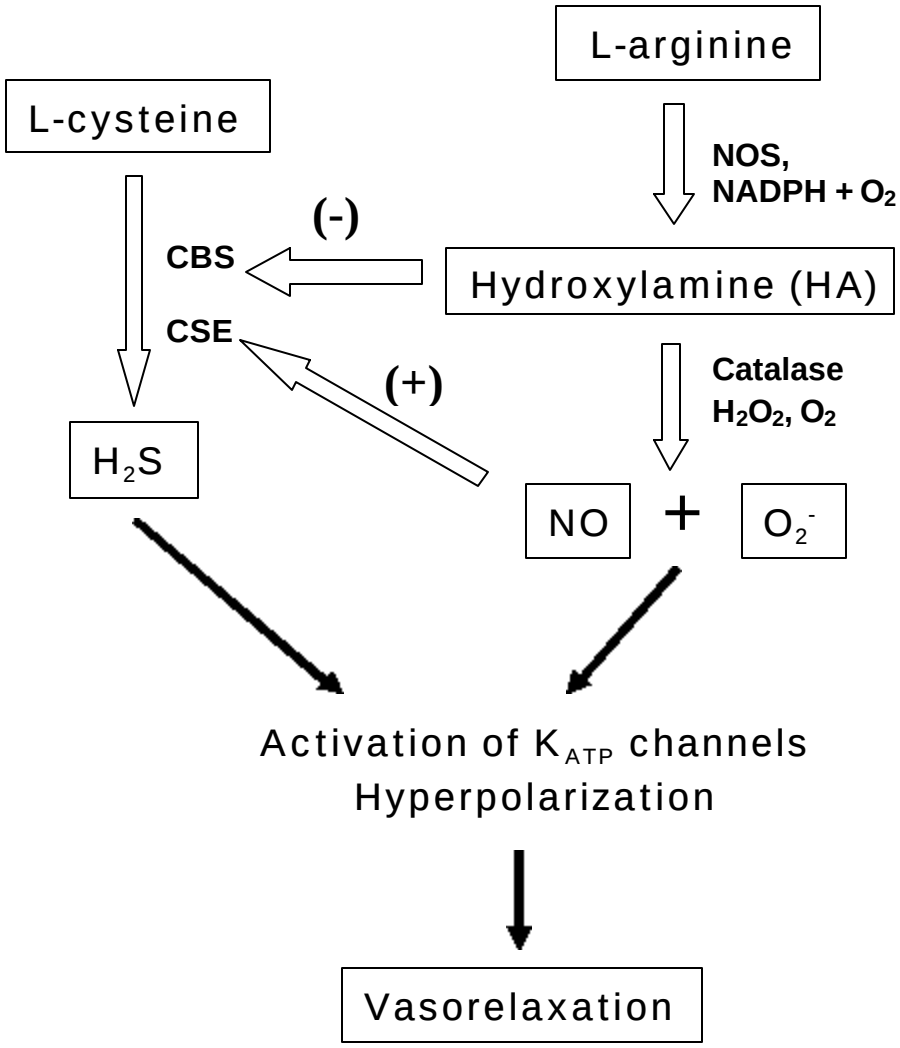


Fig. 6: Metabolic link of H_2S, NO and O_2^- pathways by hydroxylamine. Hydroxylamine (HA) is a putative intermediate in the oxidative conversion of L-arginine to NO by NO synthase (NOS), in which O_2^- is generated from HA by catalase in the presence of hydrogen peroxide (H_2O_2). HA is known as an inhibitor of H_2S-generating enzyme cystathionine â-synthase (CBS) and cystathionine ã-lyase (CSE), which decomposed L-cysteine into H_2S. The activity of CSE is upregulated by NO.

third of the total sulfide will be in the undissociated form and two-thirds as the hydrosulfide ions (US National Research Council, 1979). Thus the intact molecules of H_2S may participate in varied biological reactions. H_2S is permeable to plasma membranes as its solubility in lipophilic solvents is about five-fold greater than in water. A portion of the gas will also evaporate from solution because of low vapour pressure. Aqueous solutions containing bromine, chloride, or iodine may react with H_2S to form elemental sulfur.

1.3.4.2 The generation, metabolism, and regulation of endogenous H_2S

H_2S has been best known for decades as a toxic gas (Smith & Gosselin, 1979). Less recognized, however, is the fact that H_2S is generated endogenously and it may have a physiological role in regulating cardiovascular function. H_2S is derived from the environment and is also generated endogenously via both enzymatic and nonenzymatic pathways (Fig. 7) (Wang, 2002). Two pyridoxal-5'-phosphate-dependent enzymes, CBS and/or CSE, are responsible for the majority of the endogenous production of H_2S in mammalian tissues that use L-cysteine as the main substrate. Ammonium and pyruvate are two other end products in addition to H_2S via CBS- and/or CSE-catalyzed cysteine metabolism (Reed, 1995). CBS is the predominant H_2S-generating enzyme in liver, brain and nervous system (Kimura, 2000); while CSE is mainly expressed in vascular smooth muscle (Hosoki *et al.*, 1997; Zhao *et al.*, 2001; Wang, 2002). The expression studies using by RNase protection assay has revealed that the highest CSE mRNA level is present in pulmonary artery, followed by aorta, tail artery, and mesenteric artery (Zhao *et al.*, 2001). Furthermore, *in situ* hybridization studies have demonstrated that CSE mRNA is solely distributed to the vascular smooth muscle layer, not the endothelial

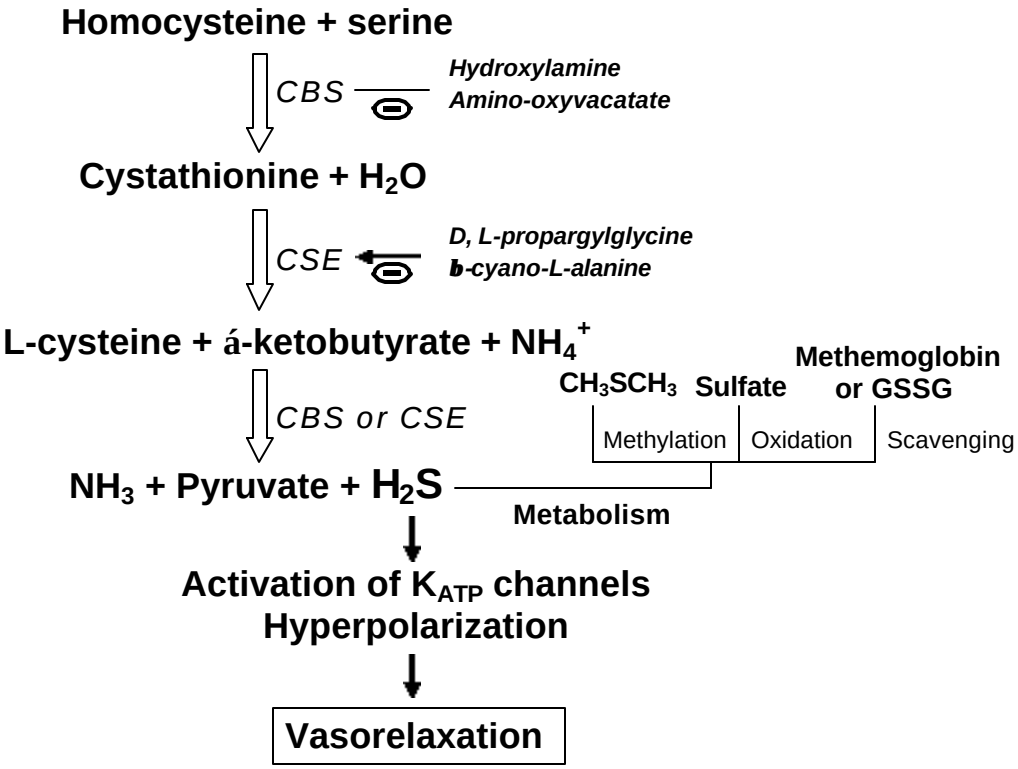


Fig. 7: Endogenous enzymatic production and metabolism of H_2S. Two pyridoxal-5'-phosphate-dependent enzymes-cystathionine â-synthase (CBS) and cystathionine ã-lyase (CSE) are responsible for the majority of the endogenous production of H_2S in mammalian tissues that use L-cysteine as the main substrate. Ammonium and pyruvate are the other two co-products of this L-cysteine metabolism. Several specific blockers for CBS and CSE are currently available. D, L-propargylglycine and â-cyano-L-alanine selectively inhibit CSE. CBS activity can be specifically inhibited by amino-oxyacetate and hydroxylamine. L-cysteine metabolites, including H_2S, ammonium and pyruvate, can not inhibit both CBS and CSE activity. H_2S *in vivo* is metabolized by the oxidation and methylation pathways. H_2S can be scavenged by methemoglobin and GSSG.

layer of rat aortic wall (Zhao *et al.*, 2001). In support of these findings, *in vitro* studies have confirmed that CSE mRNA is detected in purified and cultured VSMC, but not in cultured vascular endothelial cells. While enzymes that govern the endogenous production of NO and CO (NO synthase and heme oxygenase), are present in both VSMC and endothelial cells, the H_2S-generating enzyme appears to be located to VSMC. The expression level of CBS was undetectable in vascular tissues. CBS activity was negligible in extracts of cultured human aortic endothelial cells (Bao *et al.*, 1998; Chen *et al.*, 1999; Jacobsen *et al.*, 1995). CBS and CSE are differentially regulated. CSE expression in both yeast and mammals appears to be induced by oxidative stress (Habib *et al.*, 2000; Godon *et al.*, 1998), whereas transcription of the human CBS gene is very clearly repressed by ROS (Maclean *et al.*, 2002). Endogenous inhibitors and stimulators for H_2S production have been adequately addressed. D, L-propargylglycine (PPG) and â-cyano-L-alanine (â-CNA) selectively inhibit CSE, while CBS activity can be blocked by aminooxyacetate (AOAA) and hydroxylamine (HA). Nearly all currently available inhibitors for CBS and CSE are not membrane-permeable, which significantly impedes their applications under physiological conditions. CSE activity is increased by L-cysteine and testosterone enhances CBS activity (Stevens & Wang, 1993; Zhuo *et al.*, 1993). The expression of CBS is also inducible. Although no CBS protein could be detected in freshly isolated human aortic tissues, primarily culture of human aortic VSMC exhibited CBS activity and protein expression (Bates *et al.*, 1997). This suggests that a role for endogenous H_2S in the proliferation of VSMC that are normally quiescent. Enhancement of CBS activity by S-adenosyl-methionine (SAM) (Wang, 2001) may find novel applications with some brain disorders. However, specific activators of CSE are

not available, but these agents will serve as important tools in the regulation of abnormal cardiovascular functions related to altered endogenous H_2S metabolism.

As the end product of CBS- and CSE-catalyzed cysteine metabolism, H_2S exerts a negative feedback effect on the activity of these enzymes (Kredich *et al.*, 1973). Another less important endogenous source of H_2S is the nonenzymatic reduction of elemental sulfur to H_2S using reducing equivalents obtained from the oxidation of glucose. H_2S *in vivo* is metabolized by oxidation in mitochondria or by methylation in cytosol. H_2S can be scavenged by methemoglobin or disulfide-containing molecules such as GSSG (Wang, 2002). The elimination of H_2S from the body takes place mainly in the kidney.

Endogenous H_2S production is regulated by different mechanisms due to the tissue-specific distribution of CBS and CSE. Endogenous H_2S production in brain is regulated in three ways: i) the fast Ca^{2+}/calmodulin-mediated pathway (Eto *et al.*, 2002), ii) slow testosterone- and SAM-mediated pathway, and iii) glucocorticoid-mediated SAM synthesis (Gil *et al.*, 1997). Because CBS is the major enzyme that produces H_2S in the brain, there are 3 possibilities that may cause changes in the endogenous H_2S levels, including the levels of the substrate for CBS (L-cysteine), the amount of CBS, or the activity of CBS. Earlier, NO was suggested to upregulate the expresson of CSE in VSMC (Zhao *et al.*, 2001). The transcriptional level of CSE was significantly increased by incubating the cultured VSMCs with the NO donor SNAP for 6 h. The accumulated H_2S production during a 90-min period was also increased by incubating the homogenized rat vascular tissues with different concentrations of SNP, another NO donor.

1.3.4.3 Endogenous levels of H_2S

Endogenous level of H_2S may be determined by measuring H_2S concentration in the plasma or in tissues of interest. The H_2S level in the circulation was reported to be ~10 µM in Wistar rats (Mason *et al.*, 1978), ~46 µM in Sprague-Dawley rats (Zhao *et al.*, 2001), and 10-100 µM in humans (Richardson *et al.*, 2000). Usually the tissue level of H_2S is higher than levels in the circulation. The endogenous concentration of H_2S in rat, human, and bovine brain tissues is in the range of 50-160 µM. The endogenous level of H_2S in cardiovascular tissues has not been determined. Significant amounts of endogenous H_2S are generated from vascular tissues and this endogenous production rate of H_2S varies among different types of vascular tissues. For example, homogenates of thoracic aorta have a higher production rate than those of the portal vein of rats (Hosoki *et al.*, 1997). The production rate of H_2S in rat tail artery tissues is higher than that of rat aorta and mesenteric artery (Zhao *et al.*, 2001). When the specific inhibitor of CSE, PPG, was added to the reaction medium, H_2S production was completely abolished in all tested arteries (aorta, pulmonary, tail, and mesenteric arteries, and portal vein) (Zhao *et al.*, 2001, 2003; Zhao & Wang, 2002). This observation proves that the generation of H_2S from vascular tissue results from the specific catalytic activity of CSE.

1.3.4.4 Physiological functions of H_2S in the cardiovascular system

The cardiovascular functions of H_2S include H_2S-induced hypotension *in vivo*, muscle relaxation *in vitro*, and K_{ATP} channel activation in single VSMC. In a whole-animal study, intravenous injection of H_2S provoked a transient decrease in mean arterial pressure of rats. The H_2S-induced transient decrease in blood pressure was antagonized

by glibenclamide and mimicked by pinacidil. These data indicated that the hypotensive effect of H_2S was likely provoked by the relaxation of resistance blood vessels through the opening of K_{ATP} channels (Zhao *et al.*, 2001, 2003). H_2S-induced transient reduction of blood pressure was attributed to the scavenging of H_2S by disulfide-containing proteins, metalloproteins, heme compounds, thiol-S-methyl-transferase, and GSSG, etc; whereas the elevation of blood pressure induced by intraperitoneal injection of PPG resulted from the inhibition of endogenous H_2S production (Zhao *et al.*, 2001, 2003). This latter effect may contribute to elevated peripheral vascular resistance and increases in blood pressure.

In *in vitro* tissue studies, H_2S induced a concentration-dependent relaxation of the phenylephrine-precontracted rat aortic rings and rat mesenteric vascular beds (MAB) (Zhao *et al.*, 2001; Cheng *et al.*, 2004). The sensitivity of rat MAB to H_2S (EC_{50} of 25.2±3.6 μM) was about 5-fold higher than that of rat aortic tissues (EC_{50} of 125±14 μM). However, it is known that both vascular beds generate comparable levels of H_2S (Zhao *et al.*, 2001; 2003; Zhao & Wang, 2002; Cheng *et al.*, 2004). The higher sensitivity of MAB to H_2S emphasizes the importance of H_2S in regulating peripheral resistance and blood pressure. The removal of endothelium significantly reduced the vasorelaxation evoked by H_2S in MAB and aorta. These data suggest that the vasodilator effect of H_2S is mediated by the recruitment of endothelial relaxing factors (Zhao *et al.*, 2003; Cheng *et al.*, 2004). The H_2S-induced relaxation of MAB was partially mediated by K_{ATP} channels, because pinacidil mimicked, but glibenclamide suppressed the vasorelaxant effects of H_2S. L-cysteine, a substrate of CSE, increased endogenous H_2S production and decreased contractility of MAB. In contrast, PPG, a blocker of CSE, abolished the L-cysteine-dependent increase in H_2S production and the relaxation of

MAB. These findings indicated the importance of endogenous H_2S in regulating vascular contractility (Cheng *et al.*, 2004).

1.3.4.5 Abnormal metabolism of H_2S in the diseased state

Abnormal metabolism of H_2S may have a significant impact on cardiovascular functions. Genomic manipulations cause the altered production of endogenous H_2S and then lead to pathophysiological conditions. Defects in H_2S metabolism may be involved in central nevous system diseases.

1) Low level of H_2S: A heterozygous deficiency of CBS mice has been established (Eberhardt *et al.*, 2000). A transgenic animal model with CBS deletion causes hyperhomocystinemia, which leads to premature peripheral and cerebral occlusive arterial disease (Boers *et al.*, 1985). Homocystinuria is an autosomal recessively inherited disorder characterized, in part, by mental retardation, which maybe closely related to the low endogenous production of H_2S (Mudd *et al.*, 1989). The development of vascular disease after heart transplantation is accompanied by increased total plasma homocysteine concentration (Berger *et al.*, 1995). In such conditions of hyperhomocysteinemia, a potentially lower endogenous level of H_2S may exist. Thus, it may well be an important pathogenic factor. Patients with inherited abnormalities of the methionine metabolism exhibit significantly elevated concentrations of homocysteine and are potentially accompanied by a reduced circulating level of H_2S. These patients are prone to arteriosclerotic vascular complications during childhood. Homocysteine causes endothelial cell injury and cell detachment that initiates the development of arteriosclerosis. A lower level of circulating H_2S may also affect the structure and function of VSMC, besides homocysteine as a compounding pathogenic factor for

arteriosclerotic cerebrovascular disease. Abnormalities in the cerebral microvasculature are relevant to the cause of dementia, including Alzheimer's disease (AD). The low endogenous level of H_2S in AD brains may be caused by the decreased activity of CBS because of the lack of SAM (Morrison et al., 1996). The reduced CBS activity in patients with AD accounts for high serum homocysteine level.

2) High level of H_2S: Elevated H_2S level has been suggested to be involved in the cognitive dysfunction associated with Down's syndrome (Kamoun, 2001). Down's syndrome with elevated CBS expression, low plasma homocysteine, and significantly increased thiosulfate urinary excretion (Chadefaux *et al.*, 1988), may be coupled to abnormally high H_2S levels. These observations have led to the hypothesis that the accumulation of H_2S in the brain could cause metabolic intoxication (Kamoun, 2001). Sudden infant death syndrome may be related to abnormally high taurine levels induced by H_2S (Warenycia *et al.*, 1989).

A transgenic animal mouse model with CSE deletion needs to be developed. Whether some cardiovascular dysfunctions result from the alteration in endogenous H_2S production in CSE knockout mice remains to be established.

1.3.4.6 Activation of K_{ATP} channels by H_2S in VSMC

The before noted hypotensive and vasorelaxant responses evoked by H_2S were reduced by glibenclamide and mimicked by pinacidil, indicating the interaction of H_2S with K_{ATP} channels. Direct evidence on the stimulation of K_{ATP} channels by H_2S was derived from patch-clamp studies on single VSMC. The whole-cell K_{ATP} channel currents in rat aortic VSMC were reversibly increased by H_2S. This effect of H_2S was significantly inhibited by glibenclamide. When VSMC were exposed to H_2S, the

membrane potential of cells was increased from –36 mV to –53 mV. The increase in membrane potential by H_2S was antagonized by glibenclamide (Zhao *et al.*, 2001). It may be argued that the increase in K_{ATP} currents in the presence of H_2S resulted from altered ATP metabolism caused by H_2S. However, the fast onset of effects of H_2S on vasorelaxation and K_{ATP} channel activation and the quick reversal of effects of H_2S after the removal of the gas do not support this view. Additionally, H_2S-activated K_{ATP} currents in rat aortic VSMC were not dependent on the predetermined ATP concentration of the intracellular milieu (Zhao *et al.*, 2001). H_2S is a reductant (Kim *et al.*, 2001). It can reduce other substances and can be oxidized by O_2. In a recent study on isolated and *in vitro* perfused rat MAB, the vasorelaxant effects of H_2S were not affected by N-acetyl-L-cysteine, a potent free radical scavenger (Cheng *et al.*, 2004). Furthermore, superoxide dismutase and catalase did not alter the vasorelaxant effect of H_2S on isolated aortic tissues (Zhao *et al.*, 2001). Whether the H_2S-increased K_{ATP} channel currents can be affected by free-radical scavengers or thiyl antioxidants has not been tested. It appears that endogenous modulators of K_{ATP} channels function through cognate membrane receptors to either change ATP metabolism or alter protein phosphorylation. A direct modulation of K_{ATP} channel protein structure and K_{ATP} channel complex configuration by endogenous substances such as H_2S has been much less clear in comparison to the data available with regard to the chemical modification of K_{Ca} channels by NO. Thus, the direct interaction of H_2S with K_{ATP} channels remains to be investigated.

2. RATIONALE, HYPOTHESES, AND OBJECTIVES

2.1 Rationale

There is adequate evidence from the literature that the moment-to-moment regulation of VSMC tone is determined by changes in the activities of K^+ channels and the membrane potential. Thus, K^+ channels play a critical role in regulating the relaxation and constriction of VSMC, making a contribution to the maintenance of normal blood pressure. K_{ATP} channels couple K^+ flux and electrical activity to cellular metabolism in a variety of tissues (Quayle *et al.*, 1997). In VSMC, K_{ATP} channels have a role in the regulation of vascular tone under both physiological and pathological conditions. Rat mesenteric arteries are typical peripheral resistance arteries that participate in the regulation of systemic blood pressure (Wilson & Cooper, 1989). Nevertheless, the functional expression, the modulatory mechanism and the biophysical features of K_{ATP} channels in this resistance vessel VSMCs are largely unknown.

2.1.1 Electrophysiological and pharmacological characteristics and functional expression of K_{ATP} channels in rat mesenteric arterial VSMC

K_{ATP} channels are composed of a pore-forming subunit (Kir6.x) and a regulatory subunit (SURx). SURx closely interacts with Kir6.x and confers the sensitivity of sulphonylurea and ATP inhibition to Kir6.x (Standen *et al.*, 1989; Cook & Hales, 1984;

Inagaki *et al.*, 1996). Four K_{ATP} channel subunit genes (Kir6.1, Kir6.2, SUR1, and SUR2B) have been cloned in VSMC from rat mesenteric artery (Cao *et al.*, 2002). Kir6.1/SUR2B has claimed the title of the isoform of K_{ATP} channels in VSMC (Cao *et al.*, 2002; Fujita & Kurachi, 2000). Without knowing the expression pattern of K_{ATP} channel subunits in VSMC, the idea of a Kir6.1/SUR2B isoform in VSMC remains speculation purely based on the reconstitution study on heterologous expression systems. Whether K_{ATP} channels in resistance vessel VSMC have different roles in regulating the vascular tone and blood pressure from those in conduit vessel VSMC is unknown, particularly in the presence of H_2S and HA. Furthermore, whether K_{ATP} channel activity in resistance vessel VSMC contributes to the regulation of basal vascular tone and the resting membrane potential is unclear. The functional expression of K_{ATP} channel subunit genes (Kir6.1 and SUR2B) cloned from rat mesenteric artery VSMC and the pharmacological and electrophysiological characteristics of expressed K_{ATP} channels have never been examined.

2.1.2. Effects of H_2S on K_{ATP} channels and underlying mechanisms in VSMC

In vascular tissues, H_2S, like other gasotransmitters (Wang, 2002), may serve as a regulator of VSMC contractility. The vasorelaxant effect of H_2S was mediated by a direct stimulation of K_{ATP} channels and subsequent hyperpolarization in rat aortic VSMC (Zhao *et al.*, 2001). Since substantial differences exist between conduit and resistance arterial VSMC in functional properties such as the resting membrane potential, ionic channel currents, the role of endothelium-derived hyperpolarization factor and endothelium-dependent relaxation (Shimokawa *et al.*, 1996; Takamura *et al.*, 1999), H_2S action on conduit vessel aorta (Zhao *et al.*, 2001) can not be simply

extrapolated to peripheral resistance vessels like the mesenteric artery. However, whether H_2S elicits K_{ATP} channel activation and the resultant membrane hyperpolarization in single VSMC from rat mesenteric artery is unknown. Whether the interaction of exogenous H_2S with K_{ATP} channels is critical in determining the action mode of endogenous H_2S on K_{ATP} channels has not been determined. Modulation of K_{ATP} channel activity by the production of endogenous H_2S has not been monitored. Although important information has been collected previously using the whole-cell patch clamp technique regarding H_2S effect on K_{ATP} channels, no analysis of the changes in single-channel behaviour of K_{ATP} channels in the presence of H_2S has been conducted, in particular using unitary channel conductance and channel open probability.

2.1.3 Effects of HA on K_{ATP} channels in VSMC and its underlying mechanisms

The vasodilatory properties of HA has been documented in different vascular tissues. HA-induced vasorelaxation may be associated with the generation of HA-derived NO and O_2^- (DeMaster *et al.*, 1989; Taira *et al.*, 1997; Huang, 1998). HA also seems to coordinate the interaction between the two vasoactive gases NO and H_2S (Fig. 6), since HA inhibits CBS activity and HA-generated NO enhances CSE activity. Nevertheless, whether the vasorelaxant effects of HA involve activation of ion channels and hyperpolarization of the cell membrane has never been defined. Whether HA acts on K_{ATP} channels and membrane potential in VSMCs is not known and if HA actions are mediated by NO or O_2^- needs to be confirmed.

2.2. Hypotheses and Objectives

Three major hypotheses will be tested in this thesis:

1) Kir6.1 and SUR2B among multiple K_{ATP} channel genes cloned from rat mesenteric artery participate in the assembly of vascular K_{ATP} channels and contribute to the setting of the resting membrane potential and background K^+ conductance.

2) Endogenous as well as exogenous H_2S stimulates K_{ATP} channels in resistance VSMC independent of cGMP pathway.

3) HA modifies K_{ATP} channels via the generation of free radicals, rather than via the production of endogenous NO.

Therefore, the general objective of this thesis is to investigate the effects of exogenously applied and endogenously generated H_2S and endogenous NO donors (HA) on K_{ATP} currents and membrane potentials in VSMC and determine the underlying mechanisms. The specific goals include:

1) To characteristize the electrophysiological and pharmacological features of K_{ATP} channels in rat mesenteric artery VSMC and to determine whether K_{ATP} channels in resistance artery VSMC contribute to the setting of resting membrane potential and background K^+ conductance.

2) To examine whether homogenous or heterogenous assembly of cloned K_{ATP} channel subunit genes (Kir6.1 or/and SUR2B) can form functional channels in mammalian cell lines and to characterize the electrophysiological and pharmacological features of co-expressed K_{ATP} channels encoded by Kir6.1 with SUR2B in HEK-293 cells.

3) To examine the effects of exogenously applied and endogenously generated H_2S on macroscopic and unitary K_{ATP} currents and membrane potential in rat mesenteric artery VSMC.

4) To explore whether H_2S actions are mediated by the NO-sGC-cGMP signaling pathway or are dependent on the redox state of cysteine residues of K_{ATP} channels.

5) To examine whether intra- and extra-cellularly applied HA alters K_{ATP} channel activity and membrane potential in single VSMC.

6) To determine whether HA effects are mediated by the generation of free radicals or NO-sGC-cGMP signaling cascades.

3. MATERIALS AND METHODS

The specific techniques used in this thesis are described in detail below. The flow chart of the whole experiments is summarized in Fig. 8. Single fresh VSMC isolated from rat mesenteric arteries and cultured HEK-293 cells with and without gene transfection are visualized in Fig. 9.

3.1 Cell preparation

3.1.1 Single VSMC isolation

Single mesenteric artery VSMCs were isolated according to our previously published method with modifications (Tang *et al.*, 1999; Lu *et al.*, 2001; Zhao *et al.*, 2001). Briefly, male Sprague-Dawley rats (120-150g) were anaesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt). Small mesenteric arteries below the second branch from the main mesenteric artery were dissected and kept in ice-cold physiological salt solution (PSS) that contained (in mM): NaCl 137, KCl 5.6, NaH_2PO_4 0.44, Na_2HPO_4 0.42, $NaHCO_3$ 4.17, $MgCl_2$ 1, $CaCl_2$ 2.6, HEPES 10 and glucose 5 (pH adjusted to 7.4 with NaOH). Connective tissues were gently removed under a dissecting microscope with surgical tweezers. The freshly isolated tissues were

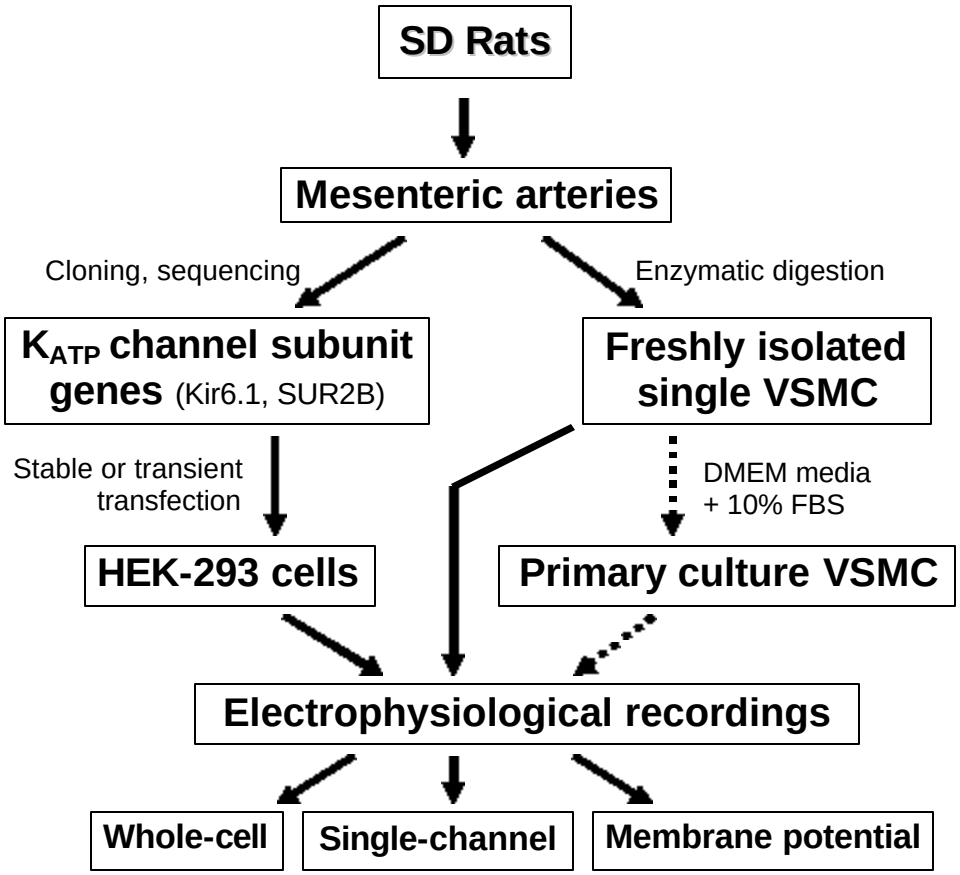


Fig. 8: Flow chart of the whole experiments. Male Sprague-Dawley (SD) rats were anaesthetized and small mesenteric arteries below the second branch from the main mesenteric artery were dissected. Tiny clean arteries without connective tissues were cut into 5 mm long pieces and then incubated with mixed enzymes for different times. After enzymatic digestion, single smooth muscle cells were released by gentle trituration and used for electrophysiological recordings including whole-cell, single-channel currents and membrane potentials. On the other hand, the main stem and first branch of mesenteric artery were dissected for molecular biological study. The vascular tissues without endothelium were homogenized to isolate total RNA, which was used as a primer to synthesize cDNA. K_{ATP} channel subunits were obtained from cDNA samples using RT-PCR. The open reading frame of K_{ATP} channel subunit genes were inserted into different vectors for cell transfection. For stable transfection, the construct containing pcDNA3.1-Kir6.1 cDNA was linearized and mixed with a FuGENE 6 transfection reagent in FBS-free RPMI-1640 medium. The mixture was added to HEK-293 cells and hygromycin selection was performed. Mock transfection (vector only transfection) was also performed. Non-transfected HEK-293 cells were included as negative control for antibiotic selection. After 5 weeks of antibiotic selective culture, viable gene-transfected cells were picked individually into culture dishes for proliferation. Construct containing pIRES2-EGFP-SUR2B cDNA was used to transiently transfect Kir6.1-stably transfected HEK-293 cells. After 48-72 h culture, cells with green fluorescence were used for electrophysiological experiments.

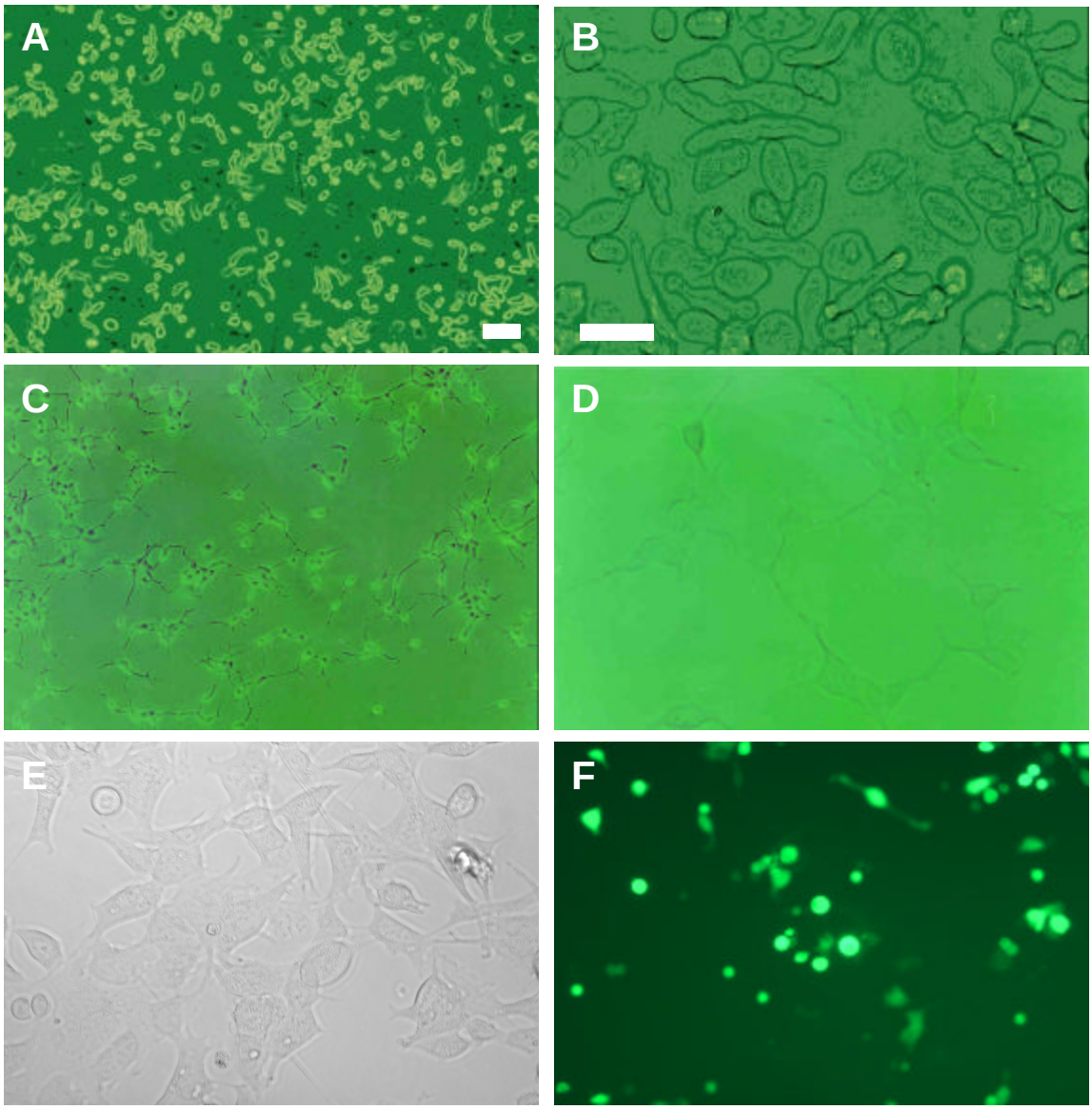


Fig. 9: Visualization of freshly isolated smooth muscle cells and non-transfected and transfected HEK-293 cells. A. Freshly isolated smooth muscle cells from rat mesenteric artery under low-power microscope (calibrator 40 µm in A and C). **B.** Freshly isolated smooth muscle cells from rat mesenteric artery under high-power microscope (calibrator 80 µm in B, D, E, and F). **C.** Non-transfected HEK-293 cells under low-power microscope. **D.** Non-transfected HEK-293 cells under high-power microscope. **E.** Bright-field image of HEK-293 cells stably transfected with Kir6.1. **F.** Green fluorescence image of Kir6.1 in HEK-293 cells transiently transfected with SUR2B. Fig. 9E & 9F were made by Cao *et al.* (2002) with the permission to use.

cut into 5 mm long pieces and then incubated for 40 min at 37°C in Ca^{2+}-free PSS containing (mg/ml) albumin 1, papain 0.5 and dithiothreitol 1, and for another 30 min in the nominally Ca^{2+}-free PSS including (mg/ml) albumin 1, collagenase 0.8, and hyaluronidase 0.8. Single cells released by gentle triturating through a Pasteur pipette exhibited a long spindle shape under a microscope. Cells were stored in Ca^{2+}-free PSS at 4°C and used on the day of isolation.

The freshly dispersed VSMC from rat mesenteric arteries were identified by inverted light microscopy according to their morphology and contractility (Tang & Wang, 2001). The intact contractile properties of fresh VSMC are indicated by the altered cellular morphology (from elongated to spherical in shape) in response to norepinephrine stimulation. The freshly dissociated VSMC with clear 3-dimensional morphology and smooth surface were employed for patch-clamp recording. However, such cells were rejected for further studies if they became flat, lost 3-dimensional structure, had membrane blebs or a rough surface, showed signs of swelling or shrinkage, or failed to attach to the bottom of the recording chamber.

3.1.2 Culture and passage of HEK-293 cells

HEK-293 cells (American Type Culture Collection, Rockville, MD) were cultured in 35 mm Petri dishes at 37 °C in a humidified incubator with 95% air and 5% CO_2 in RPMI-1640 medium containing L-glutamine and supplemented with 10% fetal bovine serum and penicillin/streptomycin. The cultured cells were subjected to gene transfection when they were grown into 70-80% confluence. After gene transfection,

cells were allowed to express for 48-72 h and became available for patch-clamp recording.

3.2 Transfection of HEK-293 cells with K_{ATP} subunit genes

3.2.1 Cloning and sequencing of the K_{ATP} channel subunits

The PCR amplified open read frame-containing K_{ATP} channel subunit genes were purified and ligated into a pCR2.1 cloning vector using a TA cloning kit (Invitrogen). The constructs were transformed and propagated in IN-áF competent cells (Invitrogen) with white/blue selection. Candidate colonies were picked up for further confirmation with suitable restriction endonucleases and then sequenced from both strands using an automatic DNA sequencer (ABI 373A, Applied Biosystem). Confirmed nucleotide sequences of K_{ATP} subunit genes were deposited into DDBJ/EMBL/GenBank databases (Cao *et al.*, 2002).

3.2.2 Stable transfection of HEK-293 cells with Kir6.1 genes

HEK-293 cells were cultured in 35 mm Petri dishes as described before (Cao *et al.*, 2002). Briefly, the constructs containing pCR2.1-Kir6.1 subunit cDNA clones (GenBank # AB043637) were cleaved with appropriate restriction endonucleases to get the cloned Kir6.1 subunit genes (with proper restriction enzyme cleavage sites at both ends). Kir6.1 gene was inserted into pcDNA3.1(-)/ hygromycin vector (Invitrogen). For stable transfection, the construct containing pcDNA3.1-Kir6.1 cDNA was linearized with Eam 1105I restriction endonuclease (MBI Fermentas). Linearized constructs were mixed with a FuGENE 6 transfection reagent (Roche) in a ratio of 1 (ìg):3 (ìl) in 100 ìl

of FBS-free RPMI-1640 medium. After incubating for 45 min at room temperature (20-22 °C), the mixture was added to HEK-293 cell in 2 ml FBS-free RPMI medium (cell density: 8×10^4/35 mm dish). Hygromycin selection was performed with concentration of 20 ìg/ml. Mock transfection (vector only transfection) was also performed. Non-transfected HEK-293 cells were included as negative controls for antibiotic selection. After 5 weeks of the antibiotic selective culturing, viable gene-transfected cells were picked individually into 24-well culture plates for proliferation. When the cells became >90% confluent in 90 mm culture dishes, they were harvested and stored for late electrophysiological studies.

3.2.3 Transient transfection of Kir6.1-stably transfected HEK-293 cells with SUR2B genes

A construct containing pIRES2-EGFP-SUR2B cDNA (GenBank # AB045281) was used to transiently transfect the Kir6.1-stably transfected HEK-293 cell line, which was grown in RPMI-1640 medium supplemented with 10% FBS in a humidified incubator with 5% CO_2 at 37^oC. Transient transfection was done without pIRES2-EGFP-SUR2B linearization using the same protocol described above. pIRES2-EGFP vector encodes a green fluorescent protein (GFP) for easy identification of transfected cells. Cells were allowed to express SUR2B for 48-72 h prior to electrophysiological experiments.

3.3 Electrophysiological recording of K_{ATP} currents and membrane potentials

3.3.1 The whole-cell K_{ATP} current recording

The conventional whole-cell patch-clamp configuration was used to record K_{ATP} channel currents in native VSMC and gene-transfected HEK-293 cells (Tang & Wang, 2001; Zhao *et al.*, 2001; Wu *et al.*, 2002). Briefly, two or three drops of cell suspension were added to the perfusion chamber inside a Petri dish that was mounted on the stage of an inverted phase-contrast microscope (Olympus IX70, Tokyo, Japan). Cells were left to stick to the glass coverslip in the experimental chamber for 5-10 min before an experiment was started. Pipettes were pulled from soft microhematocrit capillary tubes (Fisher, Nepean, ON) with tip resistances of 2-4 MΩ when filled with the pipette solution. Currents were recorded with an Axopatch 200-B amplifier (Axon Instruments, Foster City, CA, USA) and controlled by a Digidata 1200 interface. The I-V relationship and amplitude of K_{ATP} currents were constructed and measured by CLAMPFIT of pCLAMP 6.0 software (Axon Instruments). Membrane currents were filtered at 1 kHz with a four-pole Bessel filter, digitized, and stored. At the beginning of each experiment, junction potential between pipette and bath solutions was electronically adjusted to zero.

In the voltage-clamp mode, K_{ATP} channel currents of single VSMC were recorded using the conventional whole-cell patch-clamp technique. In some experiments, test pulses were made with a 10 mV increment from –80 to +70 mV with a holding potential of –60 mV. A 600 msec test pulse to different membrane potentials was applied every 10 seconds. In other experiments, voltage ramps ranging from –150 mV to +100 mV with a holding potential of –60 mV were used. A 650 msec ramp pulse

was used every 10 seconds. The sampling rate was 1 kHz. In most experiments, K_{ATP} currents were recorded at a membrane potential of –60 mV with symmetrical 140 mM K^+. The absence of Ca^{2+} and presence of EGTA in the bath and pipette solutions, and negative membrane potential of –60 mV would minimize K_{Ca} and K_V currents. The bath solution contained (in mM): NaCl 140, KCl 5.4, $MgCl_2$ 1.2, HEPES 10, EGTA 1, glucose 10 (pH adjusted to 7.4 with NaOH). The pipette solution was composed of (in mM) KCl 140, $MgCl_2$ 1, EGTA 10, HEPES 10, glucose 5, Na_2ATP 0.3, MgGDP 0.5 (pH adjusted to 7.2 with KOH). The cells were perfused continuously with the bath solution at a rate of about 2 ml/min. A complete solution change in the recording chamber was accomplished within 30 s.

In HEK-293 cells, the reconstituted K_{ATP} currents were recorded from gene-transfected cells with extracellular 40 mM K^+. A 600 msec test pulse made with a 10 mV increment from –150 to +120 mV was applied every 10 seconds. The holding potential was set at –20 mV at which the outward Kv currents were largely inactivated. The pipette solution contained (mM): KCl 107, $MgCl_2$ 1.2, $CaCl_2$ 1, EGTA 10, HEPES 5, and Na_2ATP 0.3. The bath solution contained (mM): NaCl 100, KCl 40, $MgCl_2$ 1.2, $CaCl_2$ 2.6, and HEPES 5.

3.3.2 The unitary K_{ATP} channel current recording

The inside-out configuration of the patch-clamp technique was used to record single K_{ATP} channel currents. Pipettes with a tip resistance of 4-8 MΩ were used and the seal resistance was usually greater than 10 GΩ. Membrane patches with no more than three channels were used for experiments. Single-channel currents were filtered at 2 kHz

(8-pole Bessel, -3 dB), recorded with a 100 μs sampling interval in a gap-free mode, and performed using an Axopatch 200A amplifier (Axon Instruments, Palo Alto, CA, USA). For each concentration of a tested agent, such as H_2S, glibenclamide, pinacidil or diazoxide, at least 60 s of channel activity was recorded directly on the hard disk of a computer. NP_o and the unitary current amplitude of K_{ATP} channels were determined from all point histograms using the FETCHAN and pSTAT of pCLAMP 6.0 Software (Axon Instruments). NP_o is the product of N (the number of single channels in one patch) and P_0 (the mean channel open probability) and calculated by the equation (Kajioka *et al.*, 1991). $NP_o = (A_1+2A_2+3A_3+\ldots nA_n)/(A_0+A_1+A_2+A_3+\ldots.+A_n)$. A_0, A_1, A_2, A_3 and A_n are the areas under each histogram peak when the channels are closed, one open, and simultaneous openings of 2 to n channels, respectively, assuming that all channels in the patch have the same open probability under the given condition and that they behave independently. A current level greater than 50% of the unitary channel current was considered to reflect a channel opening.

The unitary current amplitude was determined from an amplitude histogram of 15-20 s of recorded data. The histogram was fitted to a sum of Gaussian distributions by pSTAT software. The difference between two adjacent Gaussian peaks was taken as a measure of the unitary current amplitude. Because most recordings contained more than a single K_{ATP} channel, no attempts were made to study the distribution of channel dwell times. The holding potential is defined as pipette potential with reference to the ground. The single-channel currents were recorded while holding potentials were varied from –100 to +100 mV in steps of 30 mV. To establish current-voltage curves of single K_{ATP} channels, VSMC were exposed to symmetrical 140 mM K^+ solutions. Bath solution (for the intracellular side of the membrane) included (mM): KCl 120, KOH 20, $MgCl_2$ 1,

EGTA 5, Hepes 10, glucose 5, Na_2ATP 0.3, and MgADP 0.5 (pH=7.2); while pipette solution (for the extracellular side of the membrane) contained (mM): KCl 140, $MgCl_2$ 2, EGTA 2, glucose 10, and Hepes 10 (pH=7.4).

3.3.3 The membrane potential recording

In the current-clamp mode, membrane potentials of single VSMC were measured using the nystatin-perforated patch recording technique while holding the current at 0 pA (Zhao *et al.*, 2001). A stable recording of membrane potential was achieved at least 2 min after nystatin penetrated the cell membrane. The bath solution contained (mM): NaCl 140, KCl 5.4, $MgCl_2$ 1.2, HEPES 10, EGTA 2, glucose 10 (pH adjusted to 7.4 with NaOH). The pipette solution comprised (mM): KCl 140, $MgCl_2$ 1, EGTA 10, HEPES 10, glucose 5, nystatin 250 μg/ml. Because nystatin may destabilize the cell, the appearance of nystatin at the tip of the electrode was avoided by dipping the pipette tip into a nystatin-free solution and backfilling the remainder of the pipette with a nystatin-containing solution.

The pH of pipette and bath solutions will be adjusted to 7.2 and 7.4 with KOH and NaOH, respectively. All electrophysiological experiments were conducted at room temperature (20-22°C).

3.4 Chemicals and data analysis

H_2S solution was made by bubbling continuously pure H_2S gas (>99.99%) into bath solution or distilled water (50 ml) at 30°C at 100 kPa for 40 min. The final concentration of H_2S in this stock solution is 90 mM (Zhao *et al.*, 2001). H_2S stock

solution was prepared freshly on the day of experiment and then immediately diluted to the desired concentration with bath solution. The effects of H_2S on membrane potentials or K_{ATP} channel currents were recorded continuously before and after perfusing cells with H_2S-containing bath solution. Usually, a stable effect of H_2S was observed within 1-3 min of H_2S application and correspondingly recorded.

Pinacidil, diazoxide, nystatin, GDP, ATP, D,L-propargylglycine (PPG), β-cyano-L-alanine (βCNA), amino-oxyacetate (AOAA), ammonium chloride, sodium pyruvate and chloramine T (CLT), 8-bromoguanosine 3', 5'-cyclic monophosphate (8-Br-cGMP), N-acetyl-L-cysteine (NAC), superoxide dismutase (SOD), hydroxylamine (HA), sodium nitroprusside (SNP), hypoxanthine (HX), and xanthine oxidase (XO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA.); glibenclamide from Research Biochemicals International (Natick, MA, USA); and iberiotoxin from Alomone Labs (Jerusalem, Israel). Stock solutions of pinacidil, diazoxide, and glibenclamide were made in DMSO and diluted to the desired concentrations immediately prior to use. DMSO alone was without effect at the concentration used (up to 0.3%). Na_2ATP, GDP and nystatin were directly dissolved in the pipette solution to achieve the desired concentrations at the day of experiments.

All data were expressed as means ± SEM. Statistical analyses were done using paired or unpaired Student's t-test, analyses of variance in conjunction with Newman-Keuls test and analyses of variance for repeated measures where appropriate. Group differences at the level of $p<0.05$ were considered statistically significant.

4. RESULTS

4.1 Biophysical and pharmacological characteristics of native K_{ATP} channels in VSMC

4.1.1 Effects of glibenclamide on basal K_{ATP} currents and the resting membrane potentials

In the Ca^{2+}-free bath solution containing 5.4 mM K^+, the basal K_{ATP} currents were recorded and the resting membrane potentials measured in single VSMC. Bath applied glibenclamide reversibly inhibited basal K_{ATP} currents (from 148±16 pA to 72±7 pA, at +40 mV, n=6, $p<0.01$) (Fig. 10A & 10B) and depolarized the cell membrane from −48±7 mV to −36±4 mV (n=5, $p<0.05$) (Fig. 10C & 10D), indicating that basal K_{ATP} currents, sensitive to glibenclamide, contribute to the background K^+ conductance in VSMC.

4.1.2 Effects of metabolic regulators on K_{ATP} channels

K_{ATP} channels in VSMC are activated by GDP and a low concentration of ATP facilitates channel opening (Zhang& Bolton, 1995, 1996; Beech *et al.*, 1993a, 1993b). To test the sensitivity of K_{ATP} channels to metabolic regulators, GDP and ATP were used to dialyze the cells. Cell capacitance of the isolated rat mesenteric artery VSMC

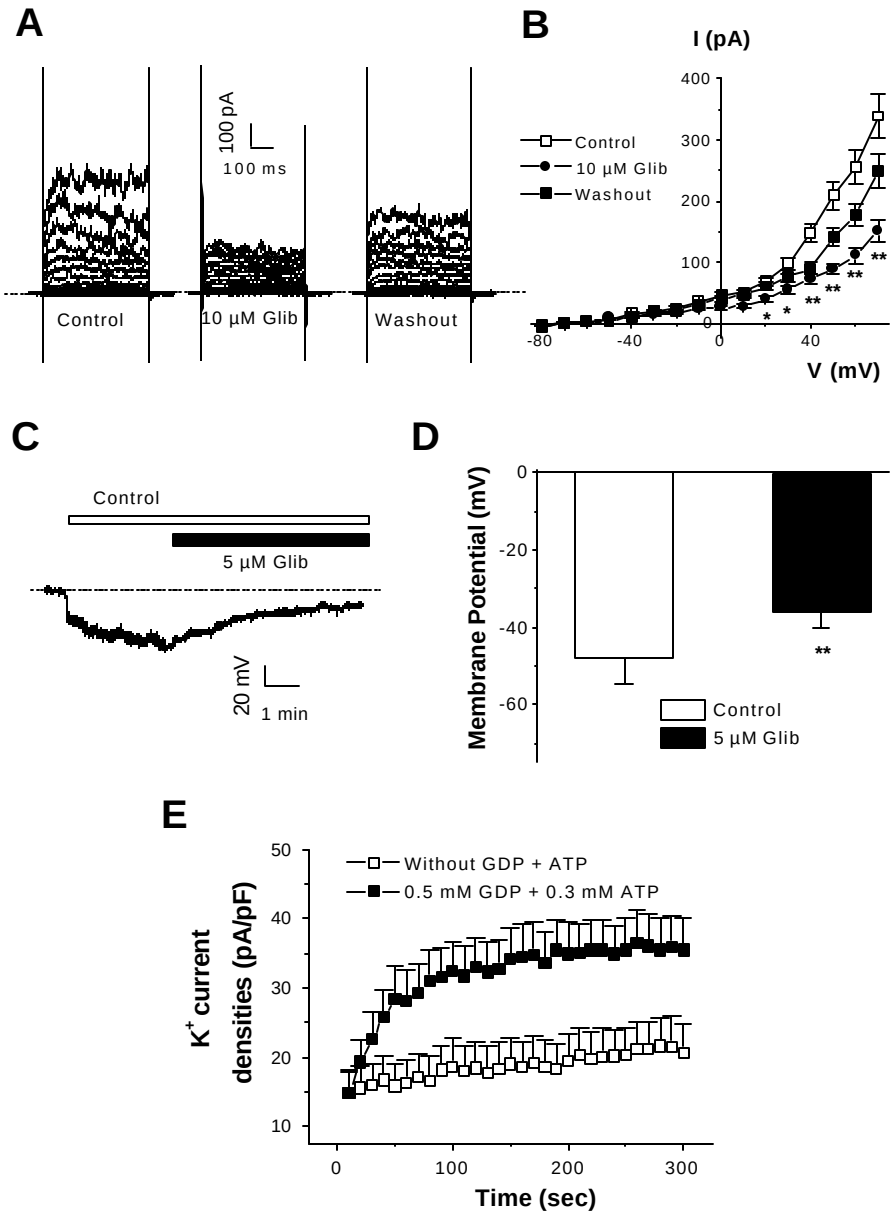


Fig. 10: The pharmacological properties of basal K_{ATP} current and the resting membrane potential in VSMC with 5.4 mM $[K^+]_o$. A. Representative original recording of basal K_{ATP} current traces in the absence and then presence of 10 μM glibenclamide (Glib). Holding potential: −60 mV; Testing potential: −80 - +70 mV. The dashed line indicates zero current. The amplitude of K_{ATP} currents was measured at 400-500 ms of each trace. **B.** The average curves of the I-V relationships. n=6, *<0.05, ** p<0.01. **C.** Representative original recording of membrane potential with the nystatin-perforated patch-clamp configuration before and after bath application of 5 μM Glib. The dashed line indicates zero potential. **D.** Summary of membrane potential changes before and after the application of 5 μM Glib. n=5, ** p<0.01. **E.** Summary of time-dependent increase of basal K^+ current densities by the dialysis of 0.3 mM ATP and 0.5 mM GDP, compared to ATP- and GDP-free conditions. HP: −60 mV; TP: +40 mV. n=8 for each group.

was 11.2±0.7 pF (n=54). The current densities of K_{ATP} channels were significantly higher with inclusion of 0.3 mM Na_2ATP and 0.5 mM MgGDP in the pipette solution than without inclusion of ATP and GDP (at +40 mV, n=8 for each group) (Fig. 10E). In order to enhance the basal K_{ATP} currents and reduce the current rundown, 0.5 mM GDP was included in the pipette solution in the following experiments together with 0.3 mM ATP. In the unitary K_{ATP} current recording, if GDP and ATP were removed from the pipette solution, no K_{ATP} channel activity appeared in an isolated inside-out patch. Thus a low concentration of nucleotides is required to maintain vascular K_{ATP} channels in the open state (Zhang & Bolton, 1996; Quayle *et al.*, 1997).

4.1.3 Effects of K^+ channel openers on K_{ATP} channels

To examine the functional role of K_{ATP} channels in VSMC, K^+ channel openers (pinacidil and diazoxide) were used to perfuse cells. With ATP and GDP in the pipette solution, basal K_{ATP} currents in VSMC were enhanced inwardly from −11±6 pA to −156±19 pA by increasing bath KCl concentrations from 5 mM to 140 mM (n=4, $p<0.01$, at –60 mV) (Fig. 11A & 11B), because the electrochemical driving force on K^+ is inward at the holding potential of −60 mV. High-K^+-amplified K_{ATP} currents were increased by pinacidil (from –156±19 pA to −286±37 pA, n=4, $p<0.05$) and then attenuated by 10 µM glibenclamide (from −286±37 pA to −76±15 pA, n=4, $p<0.01$) (Fig. 11A & 11B), but were not sensitive to external applied Ba^{2+} at 10 µM (–156±19 pA vs. −142±15 pA at –60 mV, n=4, $p>0.05$). Single K_{ATP} channel currents in inside-out patch were activated by 100 µM diazoxide and inhibited by 5 µM glibenclamide in the presence of low concentration of 0.3 mM ATP and 0.5 mM GDP (Fig. 11C). The

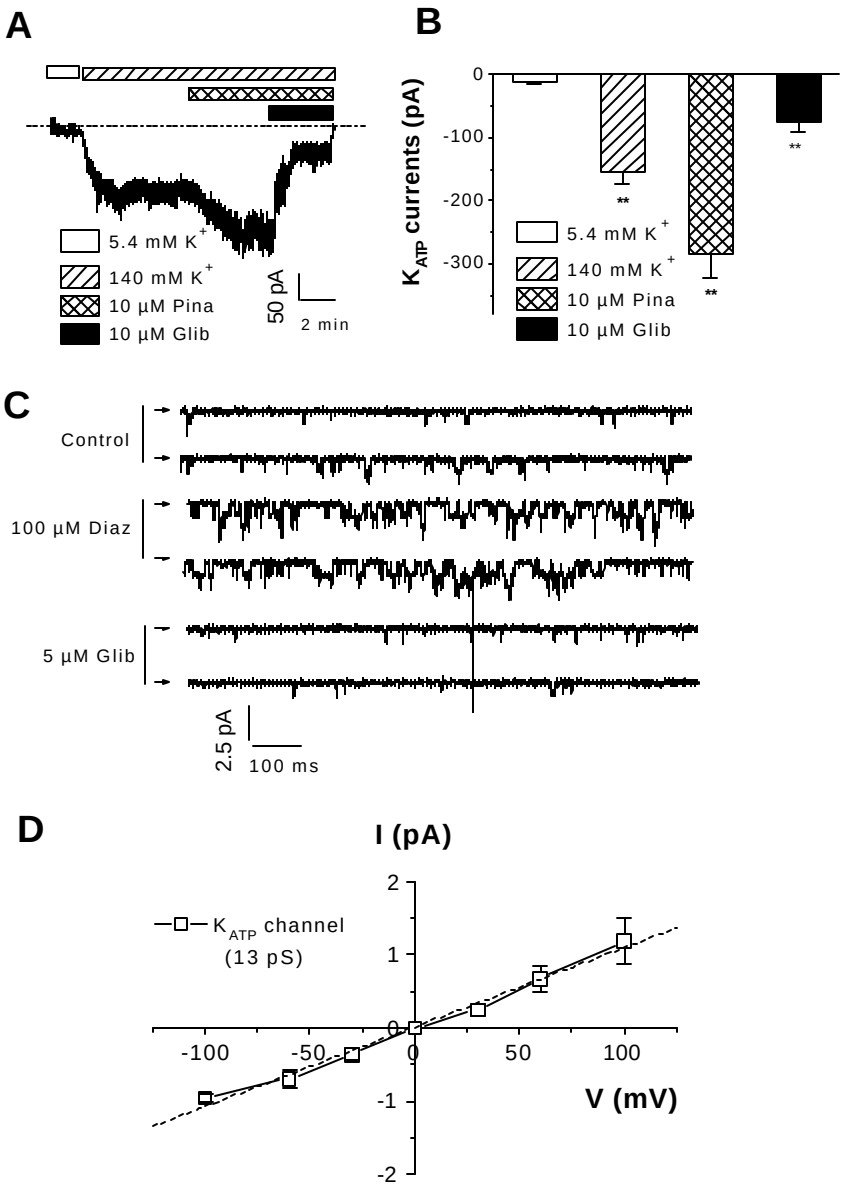


Fig. 11: The pharmacological properties of macroscopic and unitary K_{ATP} currents in VSMC dialyzed with 0.3 mM ATP and 0.5 mM GDP in symmetrical 140 mM K^+ condition. A. Representative original recording of K_{ATP} currents activated by 10 μM pinacidil (Pina) and then inhibited by 10 μM glibenclamide (Glib). Membrane potential: −60 mV. The dashed line indicates zero current. The current amplitude was measured at stable current trace for 2-5 min. **B.** Summary of K_{ATP} currents activated by Pina and inhibited by Glib. n=5 for each group, **p<0.01 (140 mM K^+ *vs.* 5.4 mM K^+; 10 μM Pina *vs.* 140 mM K^+; 10 μM Glib *vs.* 10 μM Pina). **C.** Representative original current traces of single K_{ATP} channels activated by 100 μM diazoxide (Diaz) and then inhibited by 5 μM Glib in an inside-out patch. The pipette potential is −100 mV. The arrow indicates the closed state of the channels. **D.** The I-V curve of single K_{ATP} channels showing a linear I-V relationship with a slope conductance of 13 pS. The dashed line indicates the fitting line of the I-V curve. n=5-10 for each group.

conductance of glibenclamide-sensitive K_{ATP} channels was ohmic with a slope conductance estimated at 13 pS (Fig. 11D). Furthermore, diazoxide activated K_{ATP} channels via increasing the channel open probability (NP_o from 0.08 to 0.86) (Fig. 11C).

4.1.4 K^+ selectivity of K_{ATP} channels in VSMC

To test whether K_{ATP} currents are K^+-selective, the reversal potentials of basal currents were measured and calculated in the presence of 5.4 mM and 40 mM $[K^+]_o$. With 40 mM $[K^+]_o$, inward K_{ATP} currents were increased due to an increase in the driving force acting on K^+ ions. The inward currents were stimulated and suppressed by pinacidil and glibenclamide, respectively (Fig. 12A), indicating that the recorded membrane currents under our recording conditions were mainly conducted by K_{ATP} channels. The I-V relationship curves showed that the reversal potentials were shifted from −78±2.1 mV (n=4) in 5.4 mM $[K^+]_o$ to −28±1.2 mV (n=5) in 40 mM $[K^+]_o$, quite close to the calculated K^+ electrochemical equilibrium potentials (E_K) of −80.1 mV and −32.6 mV, respectively (Fig. 12B), indicating that the recorded current is K^+-selective.

4.2 Functional expression of cloned Kir6.1/SUR2B subunit genes in HEK-293 cells and their electrophysiological and pharmacological properties

4.2.1 Basal K^+ currents in HEK-293 cells

The HEK-293 cell line has been widely used as an expression system of different K^+ channels to determine the molecular and functional expression of cloned, truncated, or mutated K^+ channel genes. However, small endogenous K^+ currents in non-

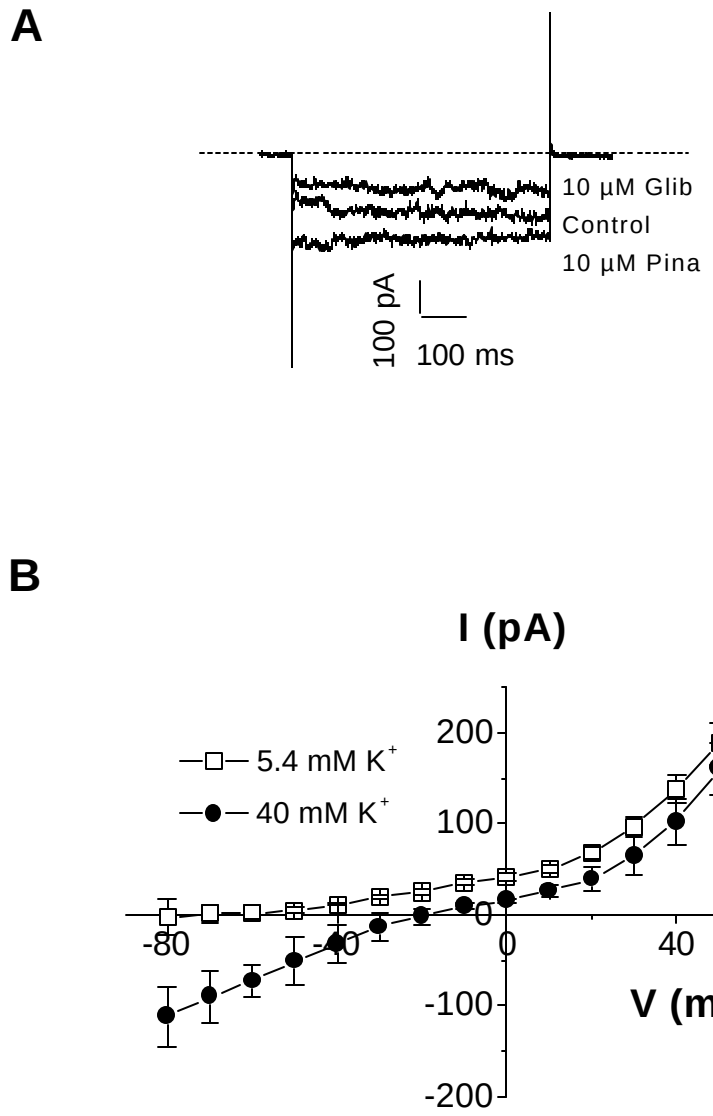


Fig. 12: The reversal potentials of K_{ATP} channels with 5.4 mM and 40 mM $[K^+]_o$ in VSMC. A. Representative original traces of inward K_{ATP} currents activated by 10 µM pinacidil (Pina) and then inhibited by 10 µM glibenclamide (Glib) with 40 mM $[K^+]_o$. Testing potential (TP)= −80 mV, Holding potential (HP)= −60 mV. The dashed line indicates zero current. **B.** The average I-V relationship curves of K_{ATP} currents with 5.4 mM and 40 mM $[K^+]_o$, showing that the reversal potentials were changed by elevating $[K^+]_o$. HP: −60 mV; TP: −80 - +50 mV. n=5.

transfected HEK-293 cells were recorded (−284±32 pA at −150 mV, n=12) with 40 mM $[K^+]_o$. To verify whether the current responses in non-transfected cells were mediated by K^+, the reversal potentials were measured at different external $[K^+]$. The increases of external $[K^+]$ from 5.4 mM to 40 mM or 140 mM shifted the reversal potential value from −81 mV to −32 mV, or −3 mM (Fig. 13A & 13B). These results were very close to the theoretical values of the K^+ equilibrium potentials of −88 mV, −34 mV, or 0 mV. The inward K^+ current was increased from −90±10 pA to −280±30 pA, and to −551±60 pA ($p<0.01$, n=5) at −150 mV with the increase of $[K^+]_o$ from 5.4 mM to 40 mM, and to 140 mM, respectively (Fig. 13C). These results showed that background K^+ currents in non-transfected HEK-293 cells were carried by K^+ ions (Jiang *et al.*, 2002).

4.2.2 Heterologous expression of K_{ATP} channel subunit genes (Kir6.1 or SUR2B alone)

Since weak inward rectification of K_{ATP} channel results from the binding of Mg^{2+} to the inner mouth of the channel pore at more depolarized membrane potential (Nichols & Lopatin, 1997), the preferred block of outward current by intracellularly applied Mg^{2+} is an important criterion to functionally identify K_{ATP} channls. HEK-293 cells transfected with Kir6.1 alone exhibited K^+ currents with a weak inward rectification of −790±60 pA (n=12, at −150 mV) with 40 mM $[K^+]_o$, compared to −292±32 pA (n=10, at −150 mV) in non-transfected cell. These K^+ currents were inhibited by 5 mM Mg^{2+} in the pipette solution (from −816±66 pA to −398±31 pA at −150 mV, n=6, $p<0.05$), and by 0.5 mM Ba^{2+} in the bath solution (from −765±56 pA to −256±29 pA at −150 mV,

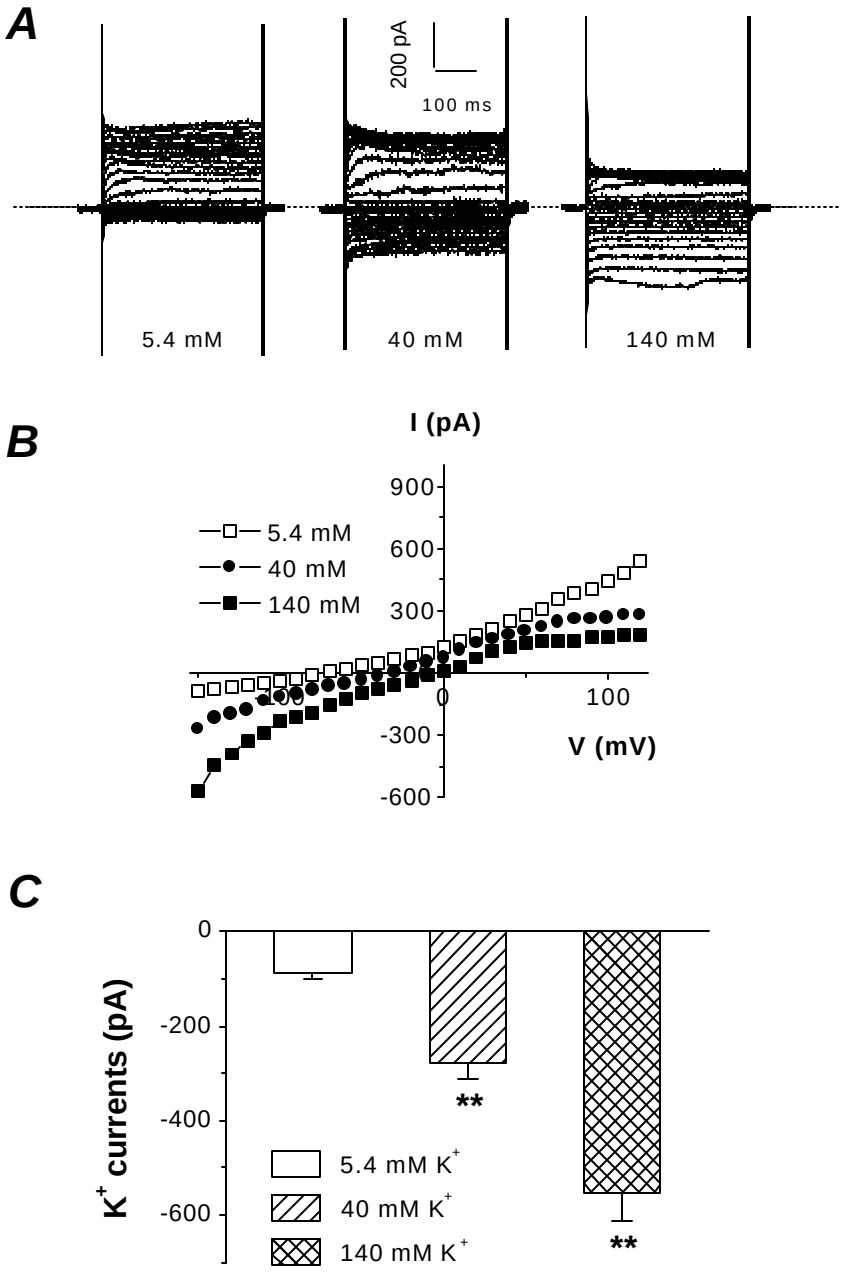


Fig. 13: The basal K^+ currents are changed by $[K^+]_o$ in HEK-293 cells. A. Representative original recording of basal K^+ currents at different $[K^+]_o$ of 5.4, 40 and 140 mM. Testing potential (TP): −150 - +120 mV. Holding potential (HP): −20 mV. The dashed line indicates zero current. **B.** The corresponding I-V relationship curves at different $[K^+]_o$. **C.** Summary of inward K^+ currents at different $[K^+]_o$. TP= −150 mV, HP= −20 mV. ** $p<0.01$ (140 mM K^+ *vs.* 40 mM K^+; 40 mM K^+ *vs.* 5.4 mM K^+). n=5 for each group.

n=7, $p<0.01$) (Fig. 14E). Mg^{2+} inhibited both inward and outward currents (Fig. 14A & 14B), while Ba^{2+} mainly blocked inward currents (Fig. 14C & 14D).

Transiently expressed SUR2B alone in HEK-293 cells also formed functional channels and elicited a weakly inwardly rectifying currents with −568±108 pA (at −150 mV, n=7) with 40 mM $[K^+]_o$ (Fig. 15A & 15B). This result was consistent with other reports that SUR1 was expressed transiently in COS-7 cells without Kir6.2 subunit (Ammala *et al.*, 1996a) and that SUR1 alone expressed in HEK-293 cells produced a dramatic increase in specific binding of [^{3}H] glibenclamide (Ammala *et al.*, 1996a). SUR2B alone-elicited currents were inhibited reversibly by glibenclamide (10 μM) from −511 pA to −190 pA and washed back to −430 pA in one cell (Fig. 15C & 15D), which were insensitive to diazoxide (n=2). Due to the limited pharmacological data on the sensitivities of these SUR2B-transfected HEK-293 cells, whether a functional K_{ATP} channel is generated by SUR2B cDNA in these cells cannot be concluded.

4.2.3 Heterologous expression of K_{ATP} channel subunit genes (Kir6.1 and SUR2B in combination)

Co-transfection of Kir6.1 with SUR2B in HEK-293 cells produced functional K_{ATP} channel currents. These co-expressed currents were stimulated by 2 mM MgADP (from −505±60 pA to −1058±130 pA, n=11, $p<0.01$) and then inhibited by 10 μM glibenclamide (to −484±65 pA, n=9, $p<0.01$) (Fig. 16). The reconstituted channels after MgADP stimulation were further increased by both diazoxide at 100 μM (from −1287±528 pA to −2170±703 pA, n=9, $p<0.01$) (Fig. 17) and pinacidil at 10 μM (from −1075±145 pA to −1662±224 pA, n=8, $p<0.01$) (Fig. 18). The K_{ATP} currents stimulated by diazoxide and pinacidil were suppressed by 10 μM glibenclamide (to −845±65 pA

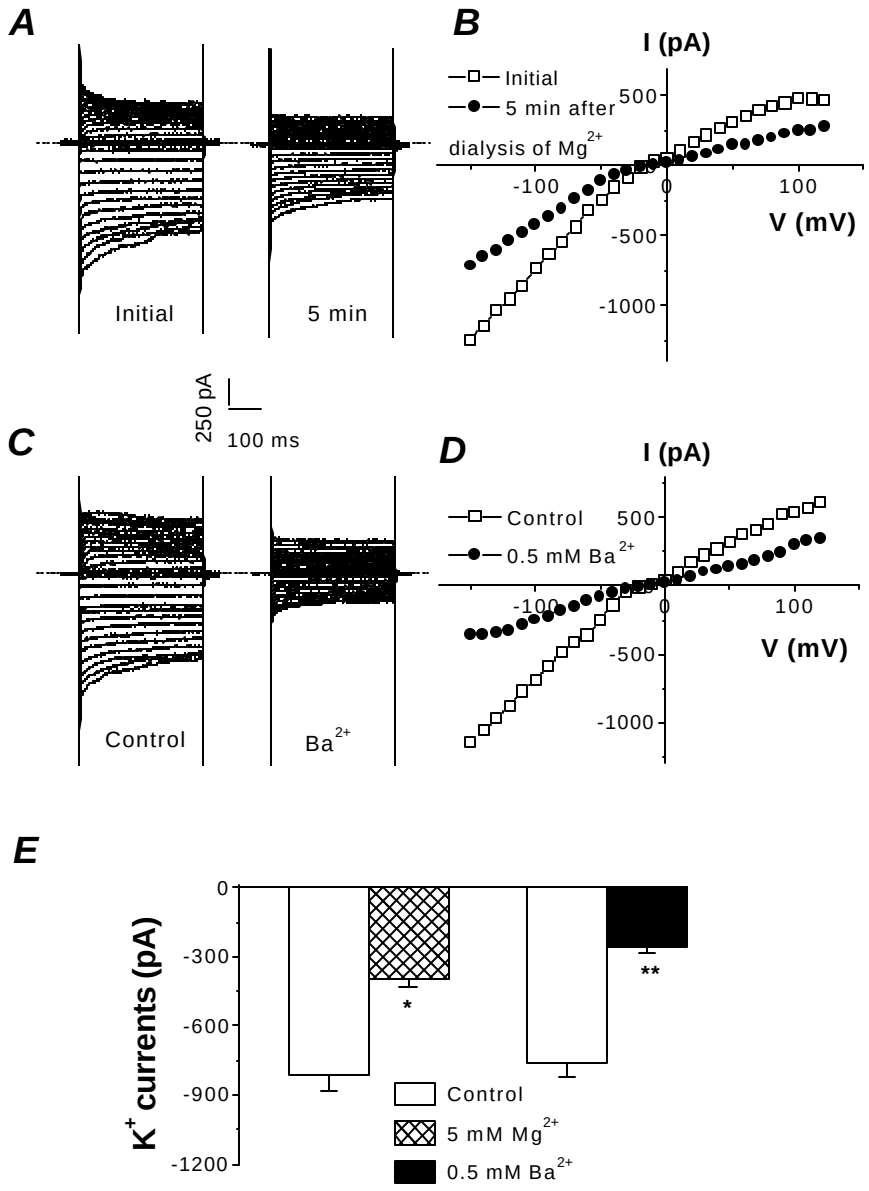


Fig. 14: Inhibition by Mg^{2+} and Ba^{2+} of the expressed Kir6.1 channels in HEK-293 cells with 40 mM $[K^+]_o$. A. Representative original recording of Kir6.1 currents at initial and 5 min after 5 mM Mg^{2+} dialysis. Holding potential (HP)= –20 mV; Testing potential (TP)= –150 - +120 mV. The dashed line indicates zero current. **B.** The corresponding I-V relationship curves of Kir6.1 currents at initial and 5 min after Mg^{2+} dialysis. **C.** Representative original recording of Kir6.1 currents before and after 0.5 mM Ba^{2+} perfusion. HP= –20 mV; TP= –150 - +120 mV. The dashed line indicates zero current. **D.** The corresponding I-V relationship curves of Kir6.1 currents before and after Ba^{2+} perfusion. **E.** Summary of Kir6.1 currents blocked by Mg^{2+} and Ba^{2+}. HP= –20 mV; TP= –150. *<0.05, **<0.01. n=6-7

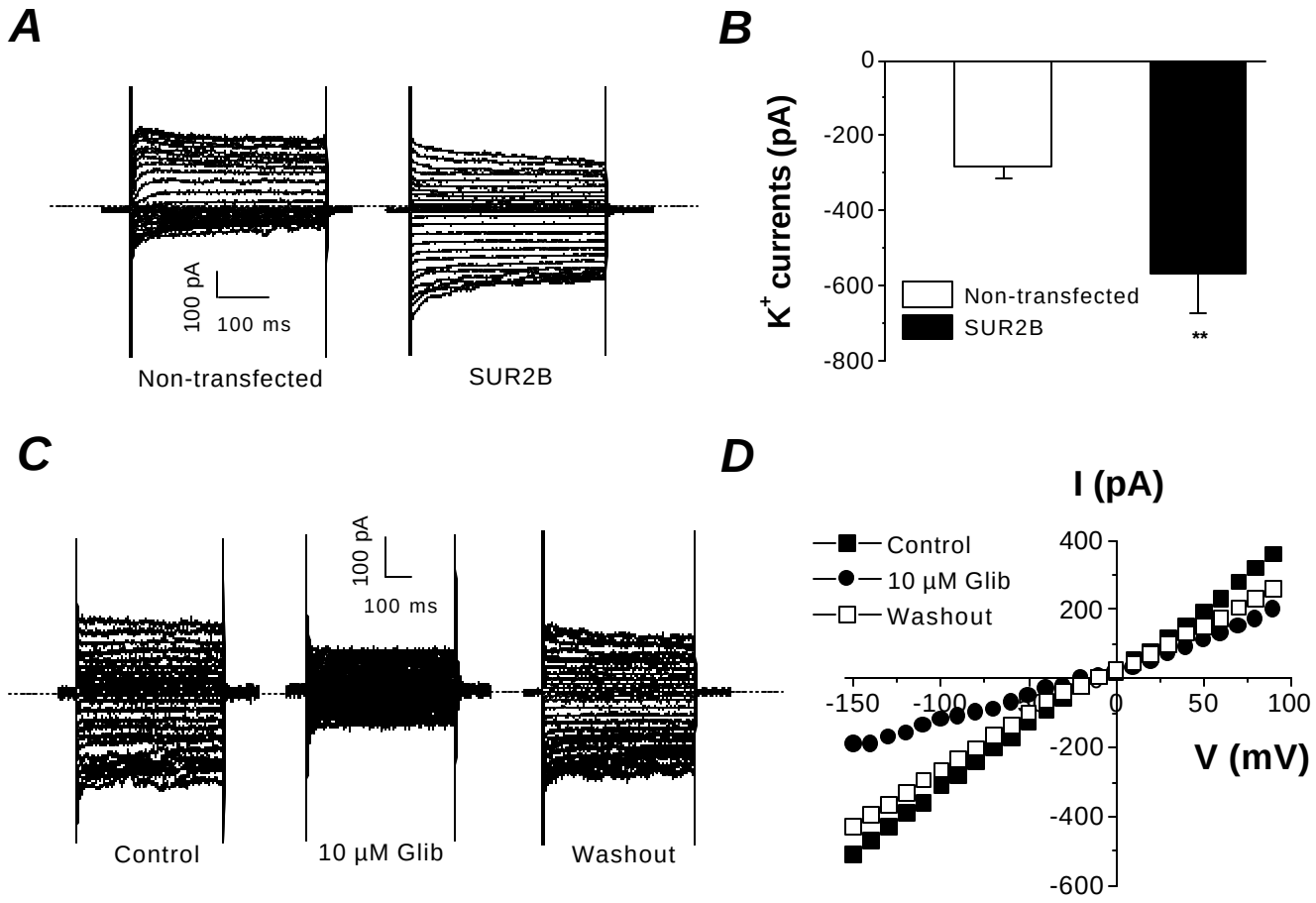


Fig. 15: Inhibition by glibenclamide of the expressed SUR2B channels in HEK-293 cells with 40 mM $[K^+]_o$. A. Representative original recording of K^+ currents in HEK-293 cells without and with SUR2B transfection. Holding potential (HP)= −20 mV; Testing potential (TP)= −150 - +120 mV. The dashed line indicates zero current. **B.** Summary of non-transfection and SUR2B-transfected currents in HEK-293 cells. HP= −20 mV; TP= −150 mV. n=7. **C.** The original recording of SUR2B currents before and after 10 µM glibenclamide (Glib) in one cell. HP= −20 mV; TP= −150 mV. The dashed line indicates zero current. **D.** The corresponding I-V relationship curves of SUR2B currents before and after Glib.

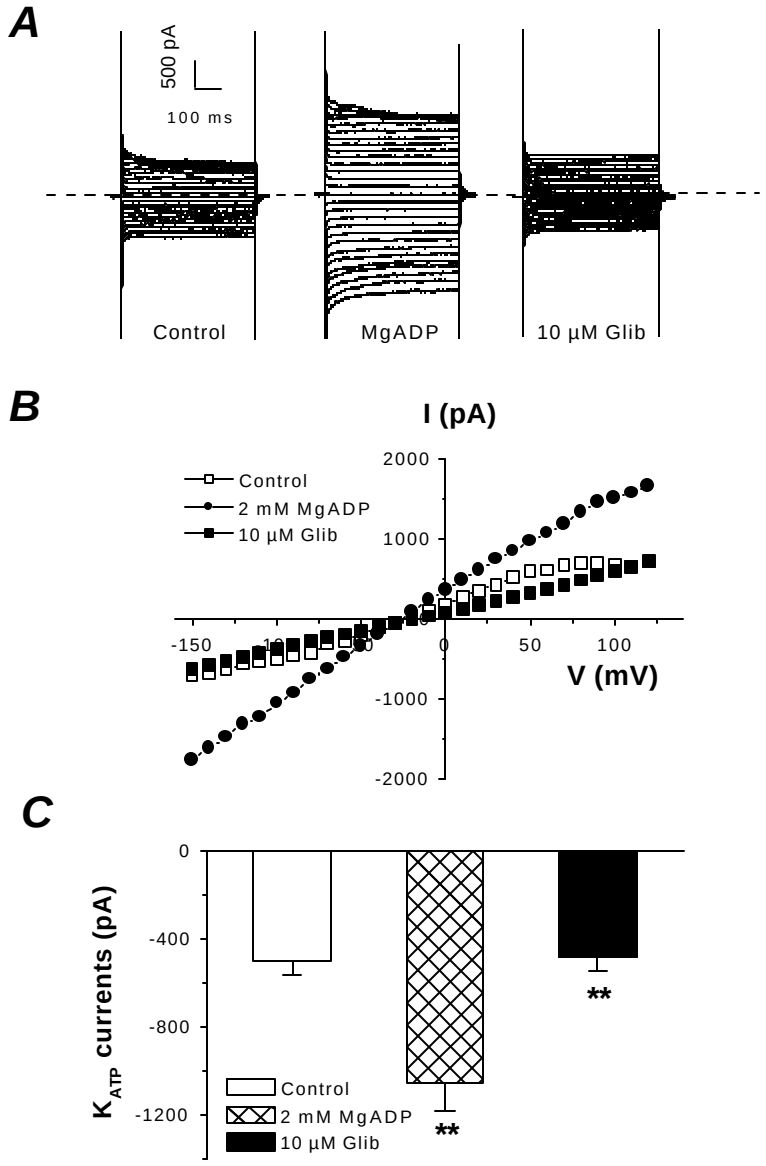


Fig. 16: Activation by MgADP and inhibition by glibenclamide of the co-expressed Kir6.1/SUR2B channels in HEK-293 cells with 40 mM $[K^+]_o$. A. Representative original recording of Kir6.1/SUR2B currents before and after 2 mM MgADP dialysis and 10 µM glibenclamide (Glib) perfusion. Holding potential (HP) = –20 mV; Testing potential (TP) = –150 - +120 mV. The dashed line indicates zero current. **B.** The corresponding I-V relationship curves of Kir6.1/SUR2B currents before and after MgADP dialysis and Glib perfusion. **C.** Summary of Kir6.1/SUR2B currents stimulated by MgADP and inhibited by Glib. HP= –20 mV; TP= –150. **<0.01 (2 mM MgADP *vs.* control; 10 µM Glib *vs.* 2 mM MgADP). n=9-11.

and −208±35 pA, respectively) (Fig. 17 & Fig. 18). The reconstituted K_{ATP} currents were inhibited by glibenclamide in a concentration-dependent fashion with an IC_{50} of 1.54±0.2 μM (Fig. 19). The channel currents with Kir6.1/SUR2B isoform were not stimulated by a low concentration of ATP (0.3 mM) (–654±102 pA *vs*. −706±122 pA, n=6), or inhibited by a high concentration of ATP (3 mM) (–784±142 pA *vs*. −742±89 pA, n=7), indicating that Kir6.1/SUR2B was insensitive to ATP inhibition.

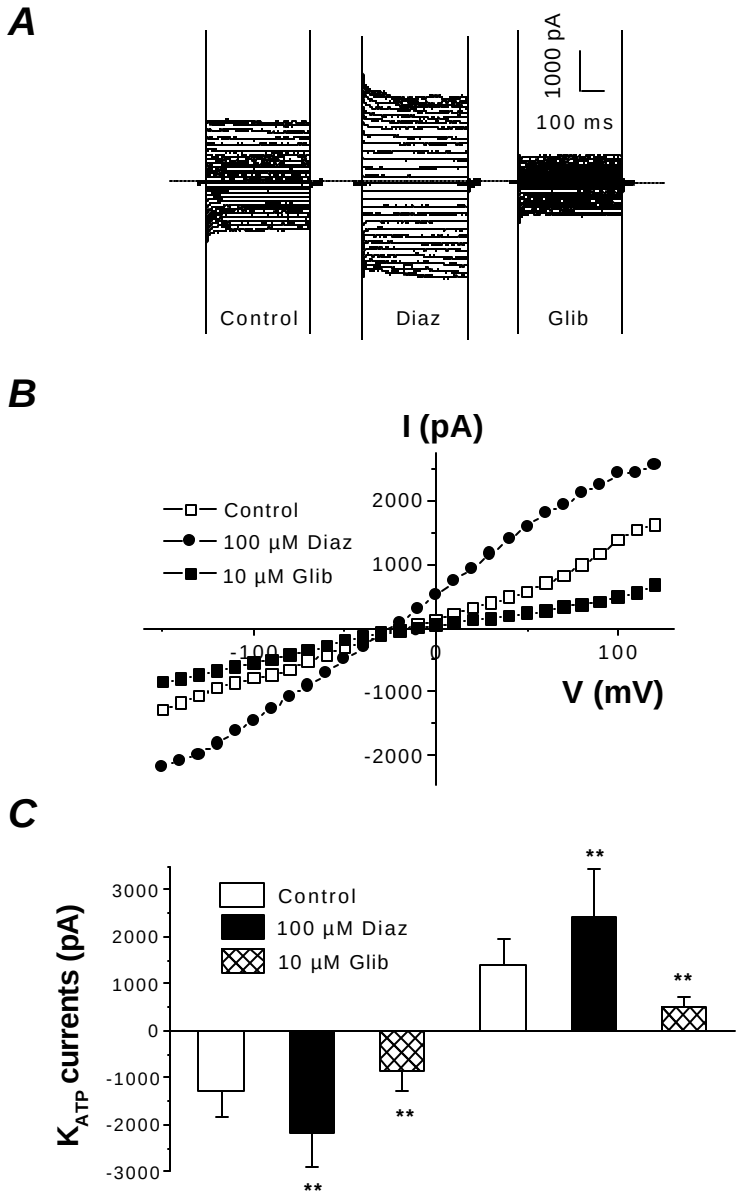


Fig. 17: Activation by diazoxide and inhibition by glibenclamide of the co-expressed Kir6.1/SUR2B channels in HEK-293 cells with 40 mM $[K^+]_o$. A. Representative original recording of Kir6.1/SUR2B currents before and after bath-applied 100 µM diazoxide (Diaz) and 10 µM glibenclamide (Glib). Holding potential (HP)= –20 mV; Testing potential (TP) = –150 - +120 mV. The dashed line indicates zero current. **B.** The corresponding I-V relationship curves of Kir6.1/SUR2B currents before and after bath-applied Diaz and Glib. **C.** Summary of Kir6.1/SUR2B currents stimulated by Diaz and inhibited by Glib. HP= –20 mV; TP= –150 and +100 mV for inward and outward currents, respectively. **<0.01 (100 µM Diaz *vs*. control; 10 µM Glib *vs*. 100 µM Diaz for both inward and outward currents). n=9 for each group.

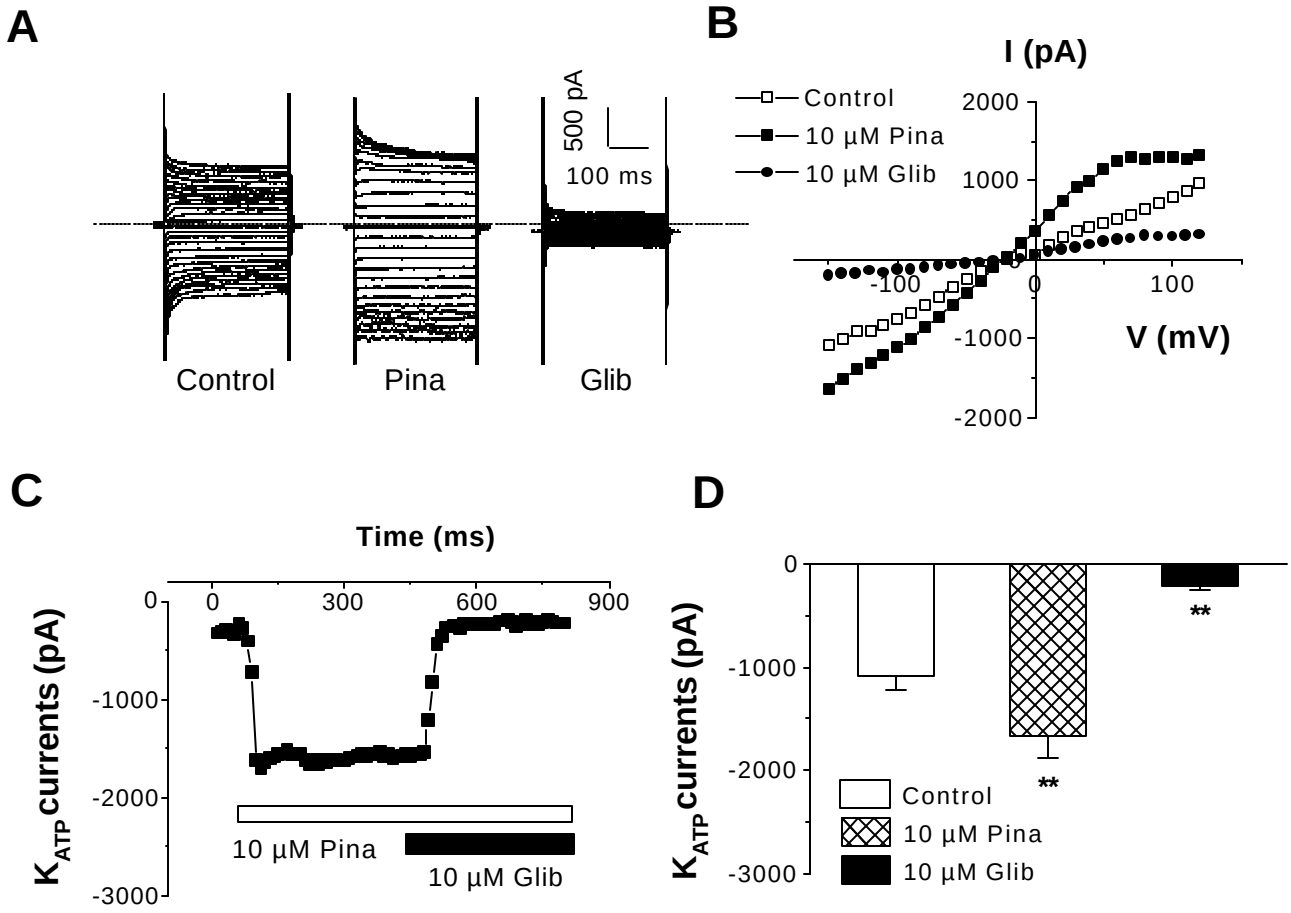


Fig. 18: Activation by pinacidil and inhibition by glibenclamide of the co-expressed Kir6.1/SUR2B channels in HEK-293 cells with 40 mM $[K^+]_o$. A. Representative original recording of Kir6.1/SUR2B currents before and after bath-applied 10 μM pinacidil (Pina) and 10 μM glibenclamide (Glib). Holding potential (HP)= −20 mV; Testing potential (TP)= −150 - +120 mV. The dashed line indicates zero current. **B.** The corresponding I-V relationship curves of Kir6.1/SUR2B currents before and after bath-applied Pina and Glib. **C.** Representative time course of Kir6.1/SUR2B currents stimulated by Pina and inhibited by Glib. HP= −20 mV; TP= −150 mV. **D.** Summary of Kir6.1/SUR2B currents stimulated by Pina and inhibited by Glib. HP= −20 mV; TP= −150 mV. n=8 for each group, **<0.01 (10 μM Pina *vs.* control; 10 μM Glib *vs.* 10 μM Pina).

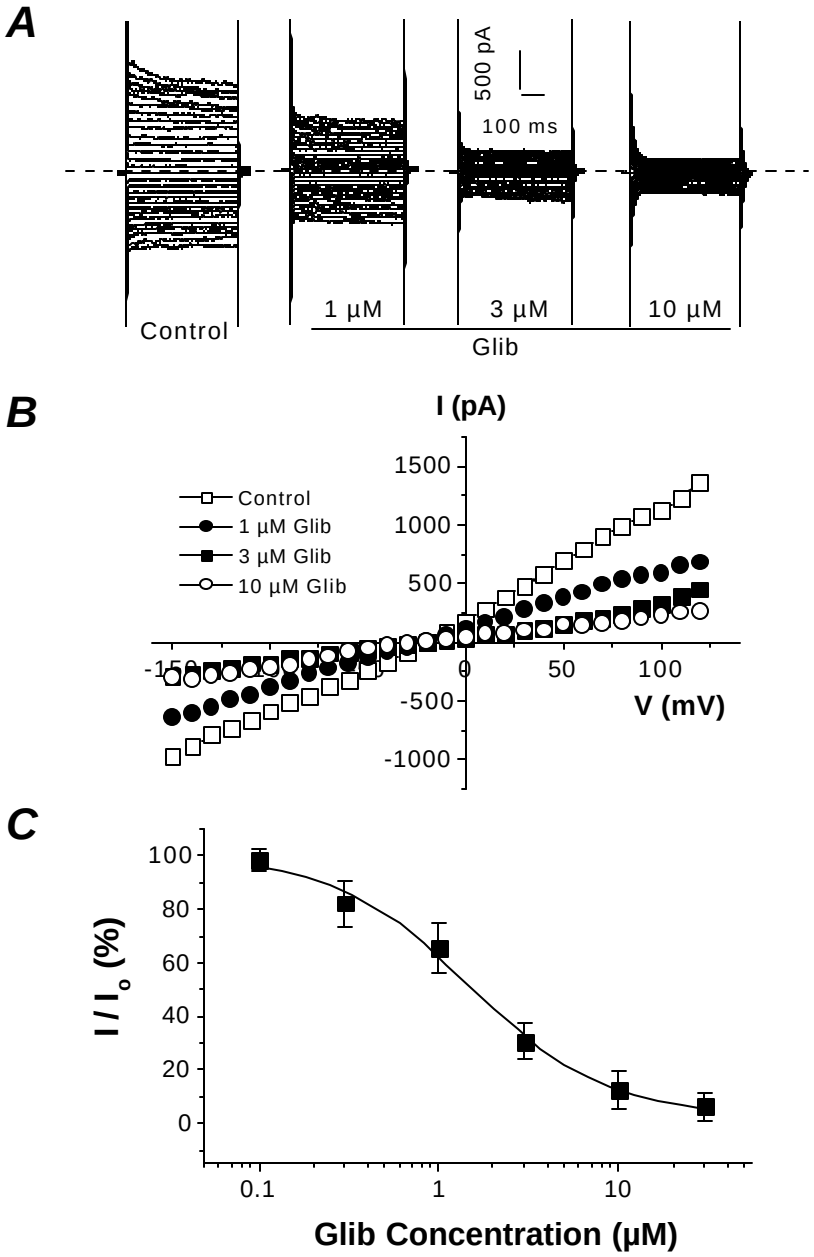


Fig. 19: The dose-effect relationship of inhibition by glibenclamide of the co-expressed Kir6.1/SUR2B channels in HEK-293 cells with 40 mM $[K^+]_o$. A. Representative original recording of Kir6.1/SUR2B currents before and after 1-10 µM glibenclamide (Glib) perfusion. Holding potential (HP) = −20 mV; Testing potential (TP) = −150 - +120 mV. The dashed line indicates zero current. **B.** The corresponding I-V relationship curves of Kir6.1/SUR2B currents before and after Glib perfusion. **C.** The dose-effect relationship of Glib on Kir6.1/SUR2B currents with an IC_{50} of 1.54±0.2 µM. HP= −20 mV; TP= −150 mV. n=6-8.

4.3 Stimulation of K_{ATP} channels in VSMC by H_2S and the underlying mechanism

4.3.1 The effects of exogenous H_2S on K_{ATP} currents and membrane potential

In symmetrical 140 mM K^+ condition, exogenous H_2S at 300 μM increased inward K_{ATP} currents in rat mesenteric artery VSMC from −108±17 pA to −222±33 pA (n=5, $p<0.01$), and then H_2S-increased K_{ATP} currents were inhibited by 10 μM glibenclamide to −74±11 pA (n=5, $p<0.01$) (Fig. 20A & 20B). H_2S stimulated the inward K_{ATP} currents in a concentration-dependent fashion with an EC_{50} of 116±8.3 μM (Fig. 20C). In nystatin-perforated cells, H_2S hyperpolarized the membrane from −46±4 mV to −58±3 mV (n=8, $p<0.01$). H_2S-induced hyperpolarization was reversed to −42±3 mV (n=8, $p<0.05$) by the removal of H_2S from the bath solution. In the same cell, glibenclamide (10 μM) further depolarized the cells to −23±2.4 mV (n=5, $p<0.01$) (Fig. 21). In inside-out membrane patches, K_{ATP} channel activity was hardly detectable with ATP-free bath solution (n=8), but increased significantly by the perfusion of 0.3 mM ATP with 0.5 mM GDP solution (n=10). Exogenous H_2S increased unitary K_{ATP} channel activity in a concentration-dependent fashion, which was blocked by glibenclamide at 5 μM (Fig. 22). Furthermore, H_2S increased NP_o of K_{ATP} channels from 0.53 to 2.67 (Fig. 22A) and from 0.31 to 1.55 (Fig. 22B), indicating that H_2S activates single K_{ATP} channel by increasing channel open probability. The I-V relationship of single K_{ATP} channels showed that unitary K_{ATP} channel conductance is 12.9±0.6 pS (n=6) in the absence of H_2S, which is similar to vascular K_{NDP} channel conductance (Zhang & Bolton, 1995;

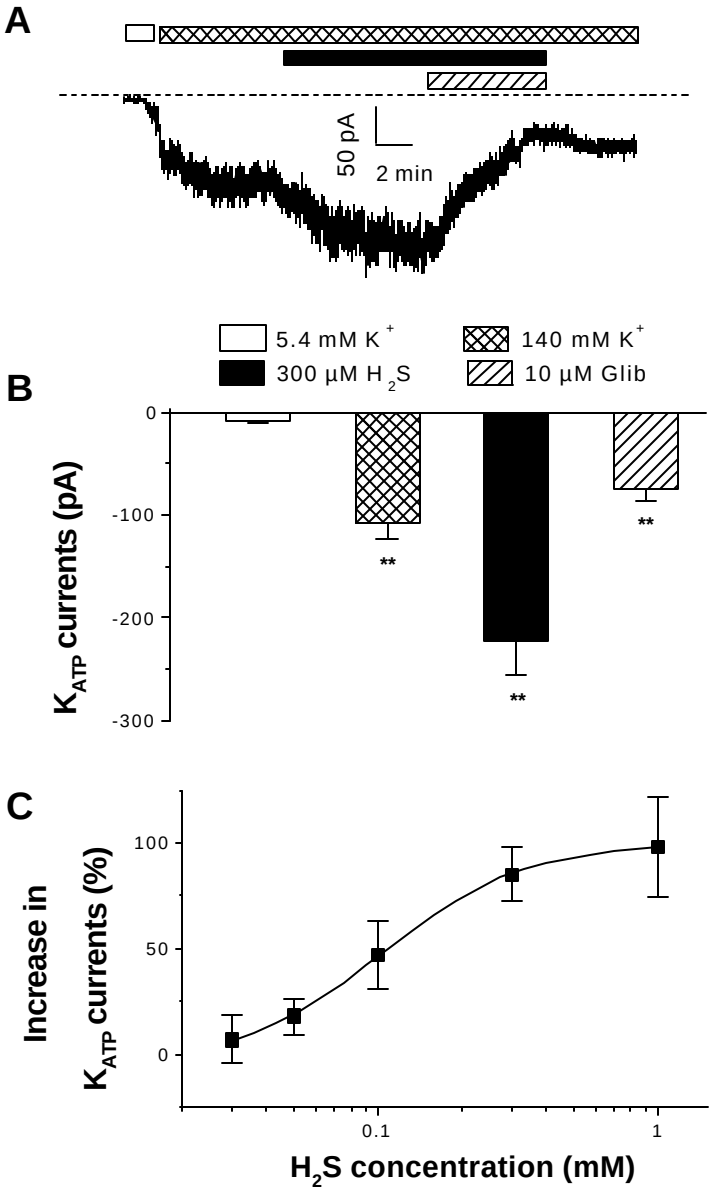


Fig. 20: The stimulatory effects of H_2S on K_{ATP} currents recorded in 140 mM symmetrical K^+ condition. A. The original current trace of K_{ATP} currents activated by 300 μM H_2S and inhibited by 10 μM glibenclamide (Glib). Membrane potential (MP): –60 mV. The current amplitudes were measured when they became stable at the maximal or minimal levels with different treatments for 0.5-1 min. The dashed line indicates zero current. **B.** Summary of the change of K_{ATP} currents activated by H_2S and inhibited by 10 μM Glib. MP= –60 mV, n=5 for each group. ** $p<0.01$ (140 mM K^+ *vs.* 5.4 mM K^+, 300 μM H_2S *vs.* 140 mM K^+, 10 μM Glib *vs.* 300 μM H_2S). **C.** The concentration-effect curve of the stimulatory effects of H_2S on K_{ATP} currents, MP= –60 mV, n=5-8.

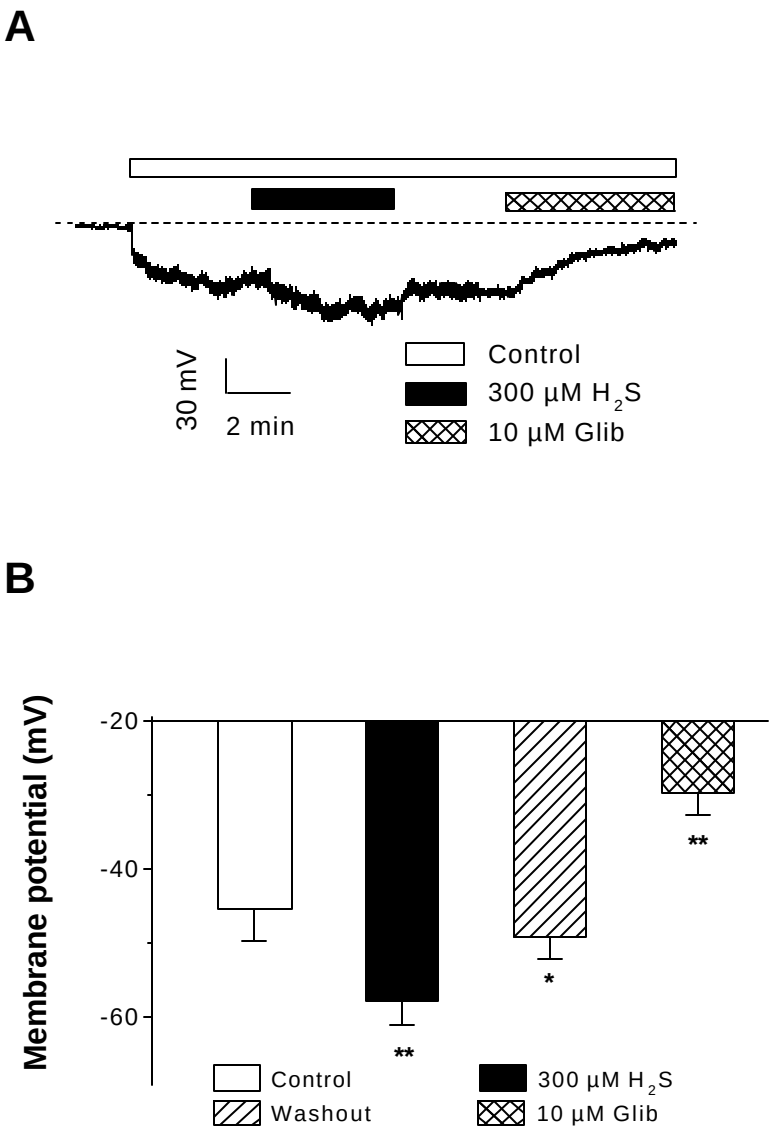


Fig. 21: The hyperpolarization of membrane potential by H_2S and its inhibition by glibenclamide in the nystatin-perforated whole-cell recording. A. The original recording trace of membrane potential increased reversibly by 300 µM H_2S and decreased by 10 µM glibenclamide (Glib). Membrane potential changes were measured when they became stable at the maximal or minimal levels with different treatments for 0.5-1 min. The dashed line indicates zero potential. B. Summary of the change of membrane potentials increased reversibly by H_2S and decreased by Glib. n=5-8. *p<0.05 (washout *vs.* 300 µM H_2S), **p<0.01 (300 µM H_2S *vs.* control, 10 µM Glib *vs.* washout).

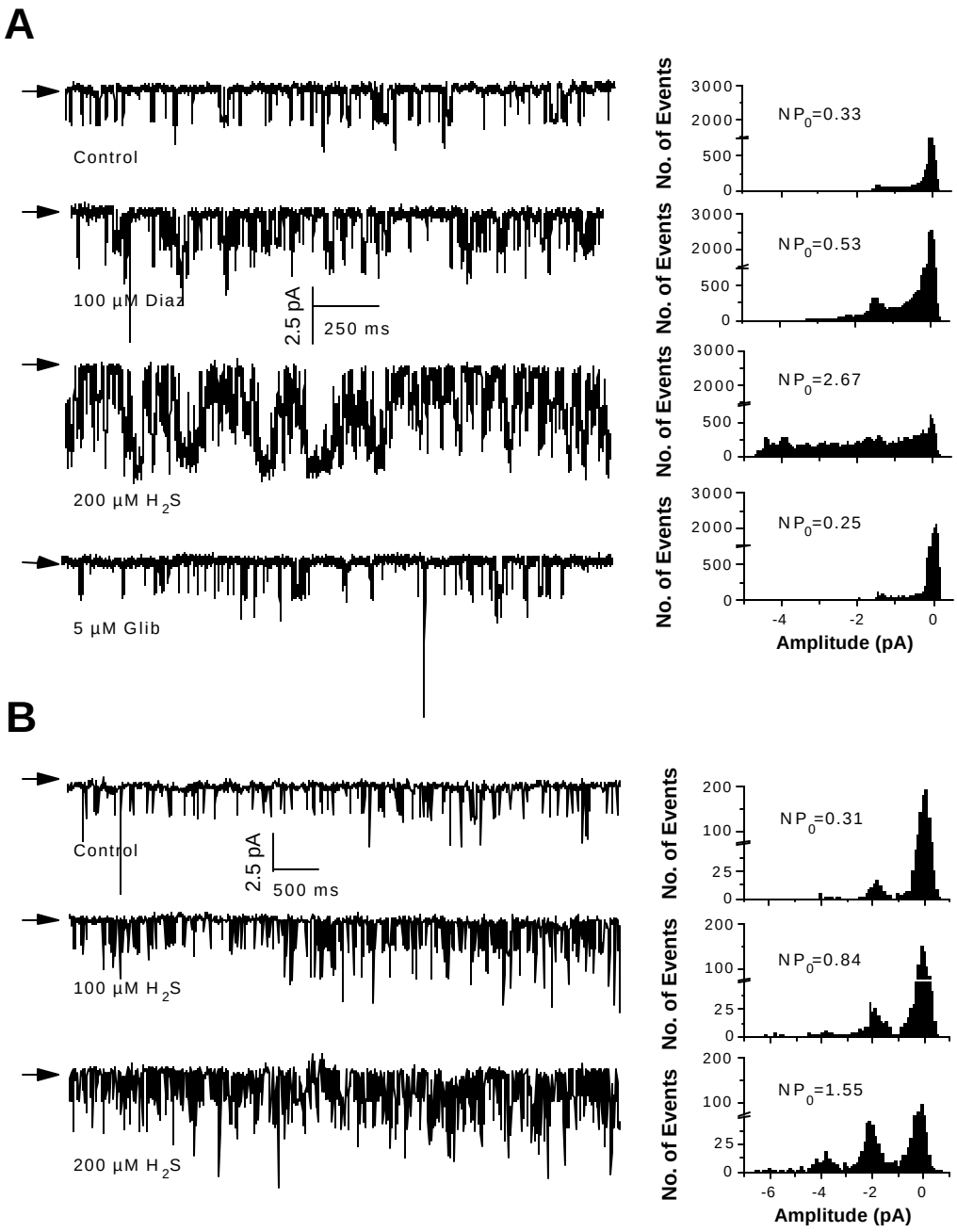


Fig. 22: H_2S stimulated unitary K_{ATP} channel activity in VSMC. A. The original recording traces of unitary K_{ATP} current activated by diazoxide (Diaz) and H_2S and inhibited by glibenclamide (Glib) in an inside-out patch with the pipette potential of −100 mV. The arrows indicate the closed state of the channels. The corresponding all-points amplitude histograms are plotted on the right with the values of NP_o. **B.** The original recording traces of unitary K_{ATP} currents activated by H_2S in two different concentrations under the same recording condition as in A. The arrows indicate the closed state of the channels. The corresponding all-points amplitude histograms are plotted on the right with the values of NP_o.

Quayle *et al.*, 1997). In the presence of H_2S, K_{ATP} channel conductance is 14.8±0.9 pS (n=5) (Fig. 23A & 23B). H_2S appeared not to affect channel conductance ($p>0.05$).

4.3.2 The effects of endogenous H_2S on K_{ATP} currents in VSMC

To determine the effects of endogenous H_2S on K_{ATP} currents, various inhibitors of H_2S-generating enzymes (CSE or CBS) were used. Single cell dialysis with 3 mM PPG caused the time-dependent inhibition of K_{ATP} currents (+ 40 mV) by 31.3%, 49.8%, 59.6% and 64.8% at 5, 10, 15, and 20 minutes, respectively (Fig. 24A). β-cyano-L-alanine (βCNA), another inhibitor of CSE, similarly inhibited K_{ATP} currents by 12.7±1.1%, 30.5±0.9%, and 55.8±1.3% at 6, 12, and 18 minutes, respectively, after dialyzing the cells (n=6) (Fig. 24B). To examine the possible involvement of CBS in vascular tissue (Zhao *et al.*, 2001), the effect of aminooxy-acetate (AOAA), a CBS inhibitor, was examined. Intracellularly applied AOAA for 10 min had no effect on K_{ATP} currents (n=5, $p>0.05$) (Fig. 24C). Two co-products of H_2S generation in L-cysteine metabolism, ammonium chloride and sodium pyruvate, also had no effect on K_{ATP} currents (n=6, $p>0.05$) when dialyzed with the pipette solution for at least 10 min (data not shown).

4.3.3 H_2S effects on K_{ATP} currents and membrane potentials are independent of the cGMP signalling pathway

The cGMP signalling pathway plays an important role in mediating the NO- and CO-induced vasorelaxation (Ignarro, 1989; Furchgott & Jothianandan, 1991; Wang *et al.*, 1997; Wang, 1998). To determine whether the H_2S-induced increase in K_{ATP}

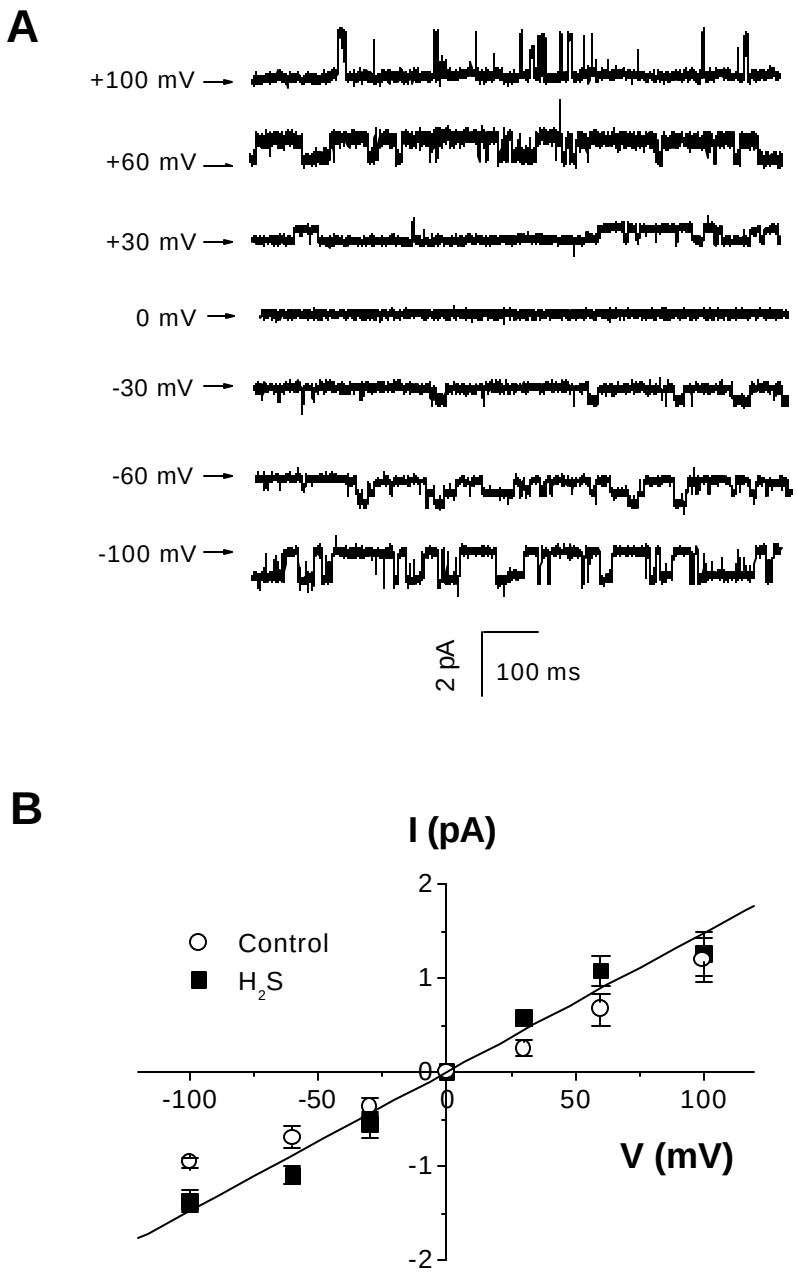


Fig. 23: Basal and H_2S-stimulated single K_{ATP} channel conductance. A. The original recording traces of basal single K_{ATP} channel current in an inside-out patch at different pipette potentials (–100 mV - +100 mV). The arrows indicate the closed state of the channels. **B.** The I-V relationships of single K_{ATP} channels with (n=5) or without (n=6) H_2S stimulation.

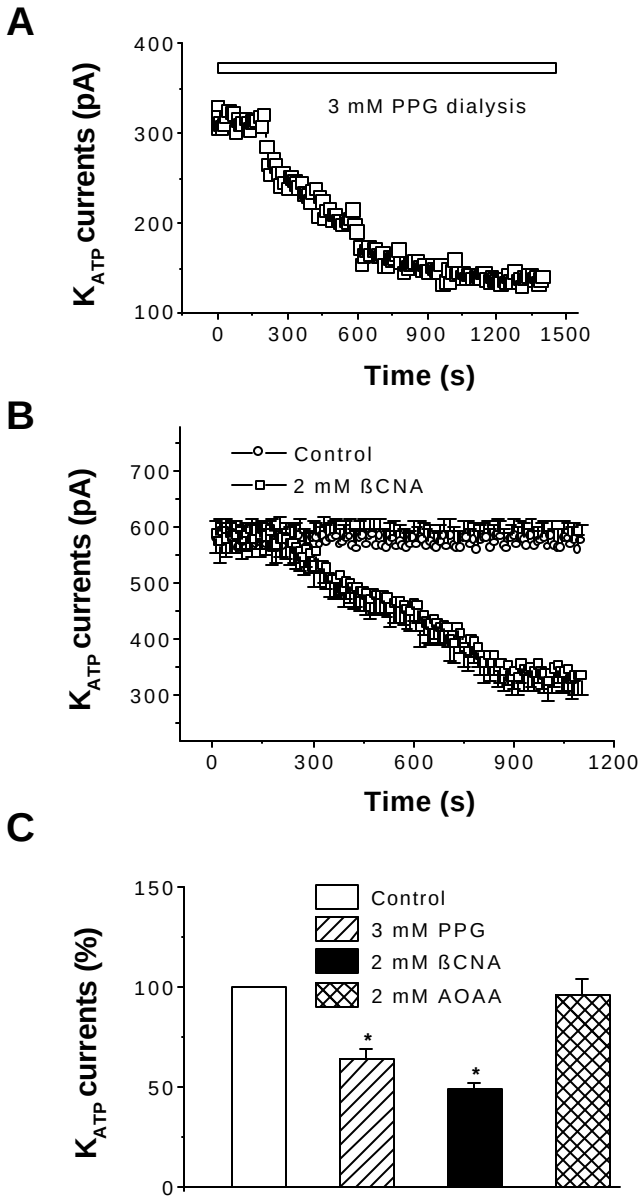


Fig. 24: The inhibitory effects of K_{ATP} channels by endogenous H_2S production inhibitors with extracellular 5.4 mM K^+ in VSMC. A. Representative time course of the inhibitory effect on K_{ATP} currents of 3 mM D, L-propargylglycine (PPG), an inhibitor of CSE, used to dialyse the cells. Testing potential (TP): +40 mV; holding potential (HP): –60 mV. **B.** Mean time course of the inhibitory effect on K_{ATP} currents of 2 mM â-cyano-L-alanine (âCNA), another inhibitor of CSE, used to dialyse the cells. TP: +40 mV; HP: –60 mV. n=6. **C.** Summary of the inhibitory effects on K_{ATP} currents of different inhibitors of H_2S-generating enzymes (CSE and CBS) in the pipette solution 10 min after the dialysis of cells. TP: +40 mV; HP: –60 mV. *p<0.05 (3 mM PPG *vs*. control; 2 mM âCNA *vs*. control). n=5-12.

currents was mediated by the cGMP pathway, we examined the effects of a membrane-permeable analogue of cGMP, 8-Br-cGMP, on K_{ATP} currents. Neither basal K_{ATP} currents nor H_2S-increased K_{ATP} currents were stimulated by 0.5 mM 8-Br-cGMP. With symmetrical 140 mM K^+, H_2S-stimulated K_{ATP} currents were not affected by 8-Br-cGMP (from −243±32 pA to −229±26 pA at −60 mV, n=6, p>0.05). Even after the application of 8-Br-cGMP was prolonged to 30 minutes or the accumulated concentration of 8-Br-cGMP was increased to 2 mM, no significant increase of K_{ATP} currents appeared. Furthermore, 8-Br-cGMP did not change H_2S-induced membrane hyperpolarization (−52±4 mV *vs.* −50±3 mV) (n=4, p>0.05).

4.3.4 Chloramine T abolished H_2S-stimulated K_{ATP} channel currents

Thiol-dependent redox mechanisms may play a role in the regulation of K_{ATP} channels (Tricarico & Camerino, 1994). Chloramine T (CLT), which oxidizes methionine and cysteine residues to form sulfoxides and sulfones (Shechter *et al.*, 1975), is known to slow the inactivation of different K^+ channels (Schlief *et al.*, 1996; Stephenes *et al.*, 1996; Ciorba *et al.*, 1997; Tang *et al.*, 2001). Bath application of CLT blocked H_2S-increased K_{ATP} currents from −271±27 pA to −98±23 pA (n=6, p<0.01) (Fig. 25A & 25B). These findings indicated that the modification of cysteine or methionine residues of K_{ATP} channel complex can abolish H_2S effect.

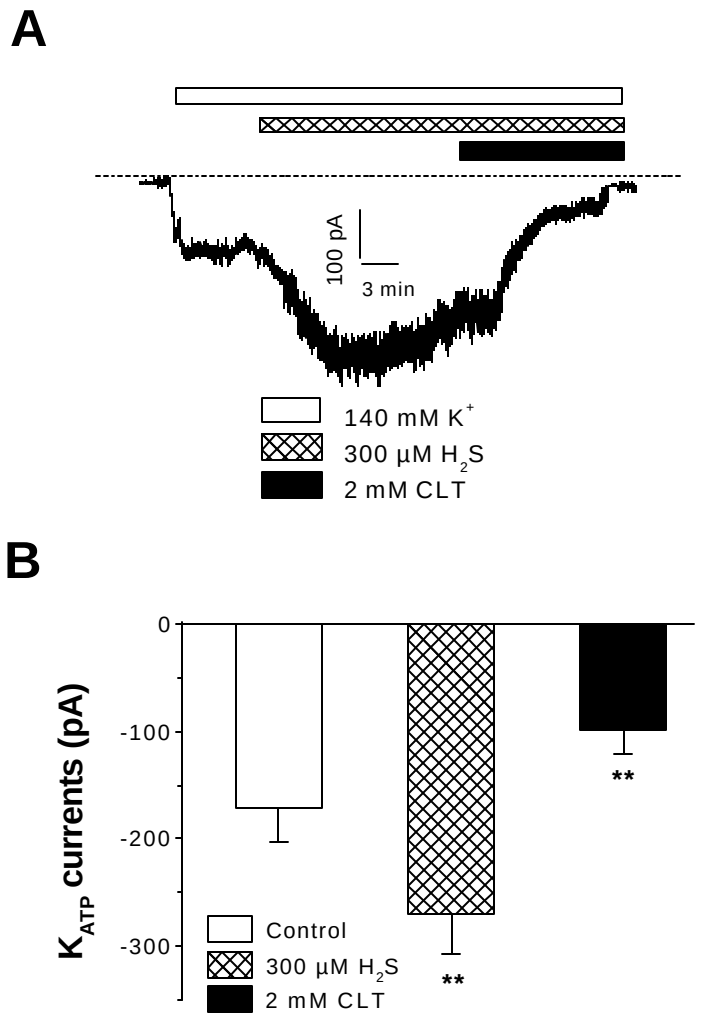


Fig. 25: Chloramine T abolished the stimulatory effects of K_{ATP} currents by H_2S in VSMC. A. The original recording trace of K_{ATP} current activated by 300 µM H_2S and abolished by 2 mM chloramine T (CLT) with symmetrical 140 mM K^+. Membrane potential (MP): –60 mV. The current amplitudes were measured when they became stable at the maximal or minimal levels with different treatments for 0.5-1 min. The dashed line indicates zero current. **B.** Summary of inhibitory effects of H_2S-stimulated K_{ATP} currents by CLT. MP= –60 mV. ** $p<0.01$ (300 µM H_2S *vs.* Control, 2 mM CLT *vs.* 300 µM H_2S), n=6 for each group.

4.4 The effects of hydroxylamine on K_{ATP} channels in VSMC and underlying mechanisms

4.4.1 HA stimulated K_{ATP} currents and hyperpolarized cell membranes in VSMC

Bath applied HA in symmetrical 140 mM K^+ condition increased K_{ATP} currents (from −180±32 pA to −380±70 pA, n=8, $p<0.01$), which were inhibited by glibenclamide to −110±13 pA (n=8, $p<0.01$) (Fig. 26A & 26B). HA activated K_{ATP} currents in a concentration-dependent fashion with EC_{50} of 54±3.4 μM (Fig. 26C). Bath-applied HA hyperpolarized the cell membrane from −48±5.2 mV to −65±7.5 mV (n=6, $p<0.01$), which was inhibited by glibenclamide to −34±3 mV (n=6, $p<0.01$) (Fig. 26D & 26E). With extracellular physiological K^+ concentration ($[K^+]_o$=5.4 mM), the whole-cell inward currents were increased by including 0.5 mM HA in the pipette solution in a time-dependent fashion (Fig. 27A). The inward currents (at −120 mV) were increased by 98±5.4%, 135±6.2% and 160±8.6% at 10, 15 and 20 min after HA dialysis, respectively (Fig. 27A & 27B). Outward currents became noisier with the increase of depolarizing stimuli (not shown). To exclude the possibility of K_{Ca} channel contamination, 200 nM IbTX, a selective K_{Ca} channel blocker, was used and it failed to prevent the HA-induced inward current increase from −195±21 pA to −255±30 pA at 10 and 20 min after HA dialysis (n=5, $p<0.05$) (Fig. 27A & 27B). After the elevation of $[K^+]_o$ to 40 mM, inward currents were profoundly increased by HA with the testing potentials of −150 to +50 mV, especially the inward current component (Fig. 28A). The inward K_{ATP} currents were increased from −320±45 pA to −684±72 pA (n=6, $p<0.01$) in a time-dependent

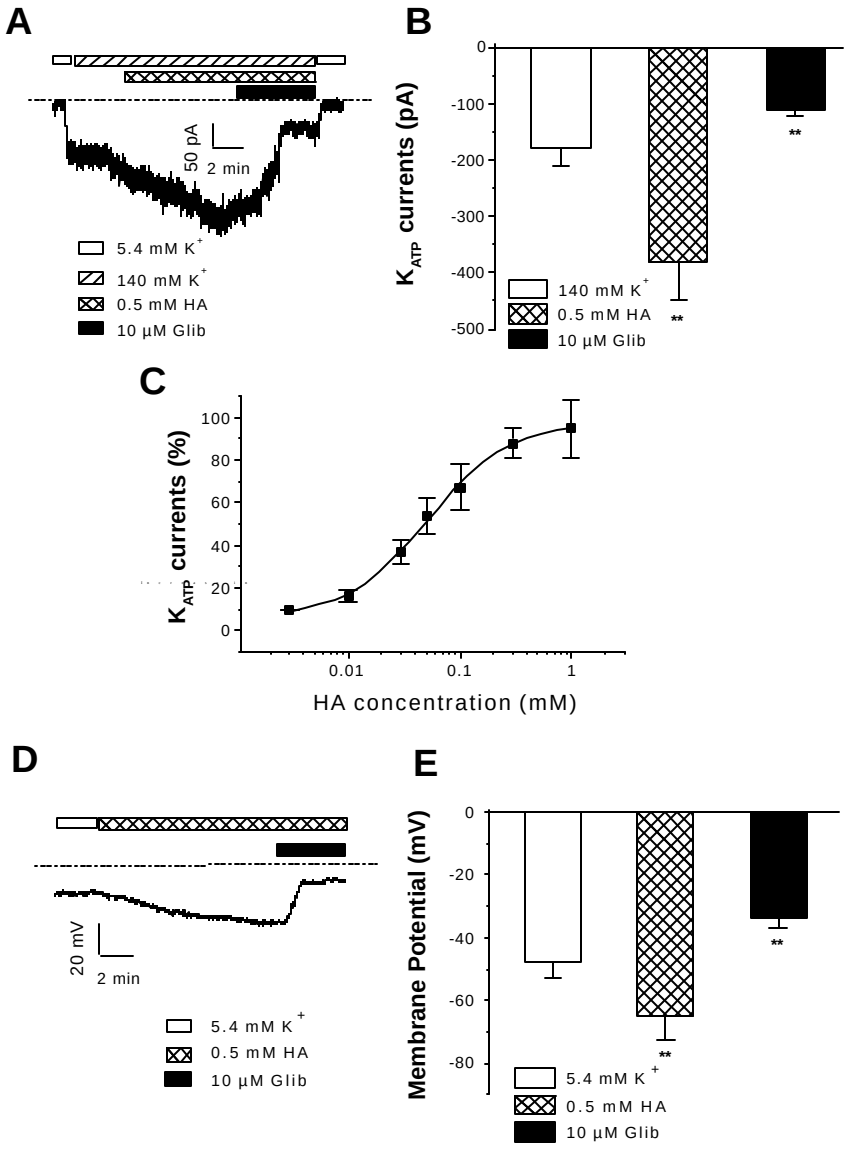


Fig. 26: Effects of hydroxylamine on K_{ATP} currents and membrane potentials in VSMC. A. Representative original current recording showing that bath-applied hydroxylamine (HA) stimulated K_{ATP} currents and these currents were inhibited by glibenclamide (Glib) in symmetrical 140 mM K^+ condition. Membrane potential (MP) = –60 mV. The current amplitude with slow activation is measured at the maximal value of different treatments for 0.5-1 min. The dashed line indicates zero current. **B.** Summary of the effects of HA and Glib on K_{ATP} currents. MP= –60 mV, ** $p<0.01$ (0.5 mM HA *vs.* control; 10 µM Glib *vs.* 0.5 mM HA), n=8 for each group. **C.** The concentration-dependent effect of HA on K_{ATP} channel currents in symmetrical 140 mM K^+ condition. MP= –60 mV, n=5-7. **D.** Representative original recording of membrane potential changes induced by HA and Glib with 5.4 mM $[K^+]_o$. The potential amplitude is measured at the maximal value of different treatments for 0.5-1 min. The dashed line indicates zero potential. **E.** Summary of the effects of HA and Glib on membrane potentials. ** $p<0.01$ (0.5 mM HA *vs.* 5.4 mM K^+; 10 µM Glib *vs.* 0.5 mM HA). n=6 for each group.

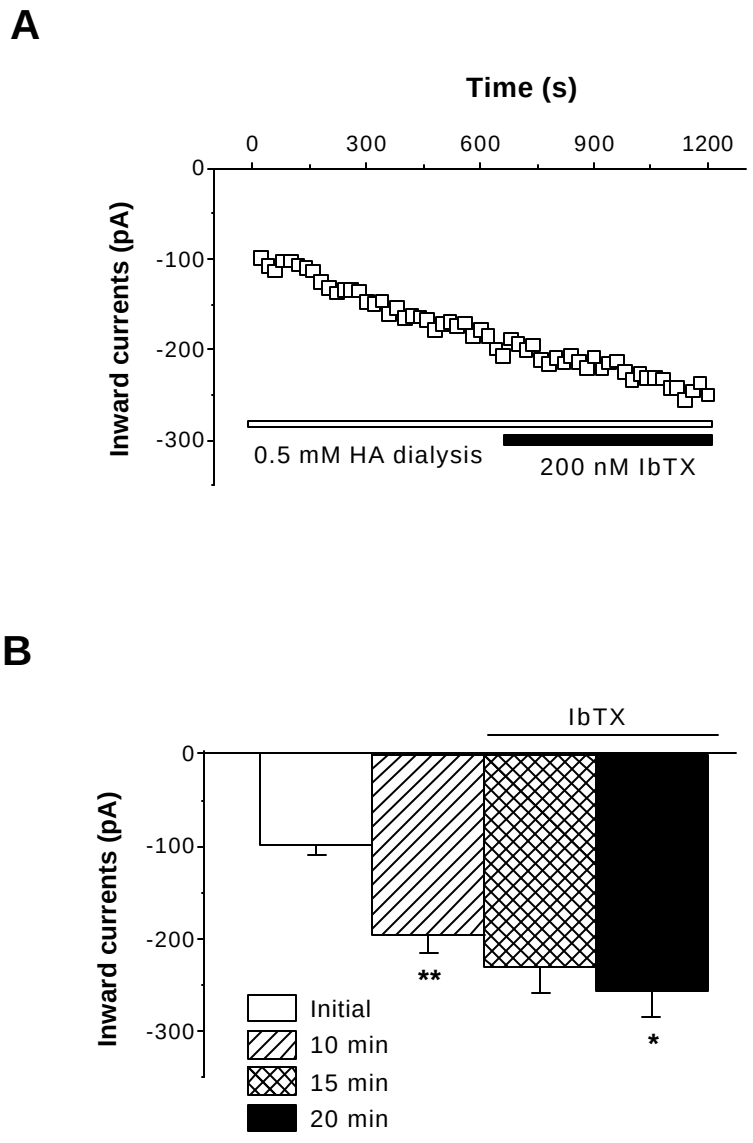


Fig. 27: Hydroxylamine stimulated K_{ATP} currents in VSMC with extracellular 5.4 mM K^+. A. The representative time-dependent effect of the intracellularly applied 0.5 mM hydroxylamine (HA) on K_{ATP} currents in the absence and then presence of 200 nM iberiotoxin (IbTX) in the bath solution. The testing potential was set at –120 mV for inward current with HP of –20 mV. **B.** The effect of IbTX on K_{ATP} currents at different time points after the dialysis of cells with HA. HP= –20 mV. n=5 for each group, * $p<0.05$ (20 min *vs.* 10 min), ** $p<0.01$ (10 min *vs.* initial)

fashion after HA dialysis (Fig. 28B & 28C). However, HA-increased currents were not significantly inhibited by extracellularly applied Ba^{2+} at 10 μM (−657±45 pA *vs.* −624±52 pA at −150 mV, n=5), but were blocked by the high concentration of Ba^{2+} at 0.5 mM (from −624±52 pA to −334±22 pA at −150 mV, n=5, $p<0.01$).

4.4.2 Effects of free radical generating system and scavengers on K_{ATP} currents in VSMC

To determine the involvement of free radicals in HA-induced effects, a free radical generation system, HX/XO, was applied to VSMC. With 5.4 mM K^+ in the bath solution, basal K_{ATP} currents recorded by a ramp pulse were also increased by HX/XO (100 μM/20 mU/ml) by 118% (at −120 mV), which was blocked by SOD by 60% (Fig. 29A & 29B). In symmetrical 140 mM K^+ solutions, the combined application of HX at 100 μM and XO at 20 mU/ml stimulated HA-elicited K_{ATP} currents at −60 mV (from −355±40 pA to −480±62 pA, n=6, $p<0.05$), which was blocked by 500 U/ml SOD (to −150±20 pA, n=6, $p<0.01$) (Fig. 29C & 29D). On the other hand, the bath-applied HA stimulated K_{ATP} currents in symmetrical 140 mM K^+ solutions from −250±26 pA to −380±45 pA (n=4, $p<0.05$), which was inhibited by SOD to −160±20 pA (n=4, $p<0.01$) and further inhibited by glibenclamide to −45±3 pA (n=4, $p<0.01$) (Fig. 30A & 30B). K_{ATP} currents stimulated by the bath-applied HA were inhibited reversibly by 300 and 600 μM NAC by 48±5% (n=5, $p<0.01$) and 61±9% (n=5, $p<0.01$) respectively, and also inhibited by SOD by 43±6% (n=5, $p<0.05$) (Fig. 30C & 30D).

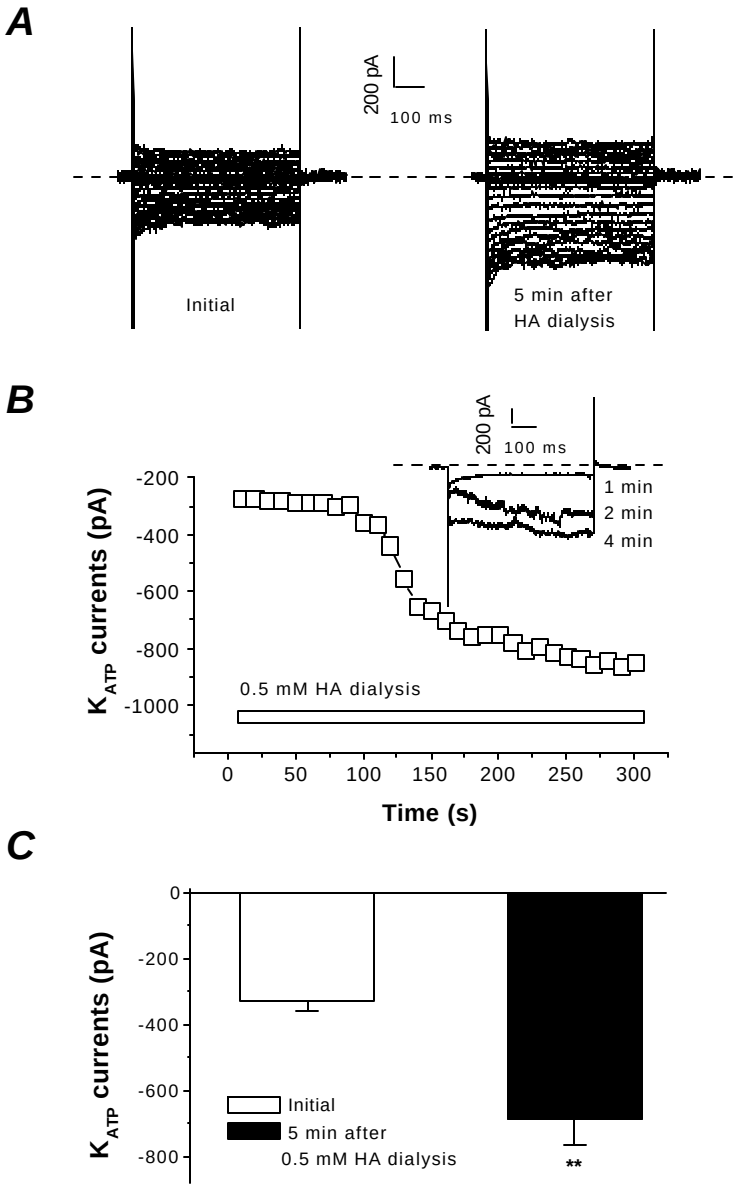


Fig. 28: Hydroxylamine stimulated K_{ATP} currents in VSMC in extracellular 40 mM K^+ conrition. **A.** The original current recordings on the effect of hydroxylamine (HA) dialysis on K_{ATP} currents. The dashed line indicates zero current. Holding potential (HP) = –20 mV, testing potential (TP)= –150 - +50 mV. **B.** The representative time-dependent effect of HA dialysis on K_{ATP} currents with HP of –20 mV and TP of –150 mV. The inset presents the original current traces of HA-increased K_{ATP} currents 1, 2 and 4 min after HA dialysis. The dashed line indicates zero current. **C.** Summary of HA-increased K_{ATP} currents at initial and 5 min after the dialysis of 0.5 mM HA. HP= –20 mV and TP= –150 mV. **p<0.01. n=6 for each group.

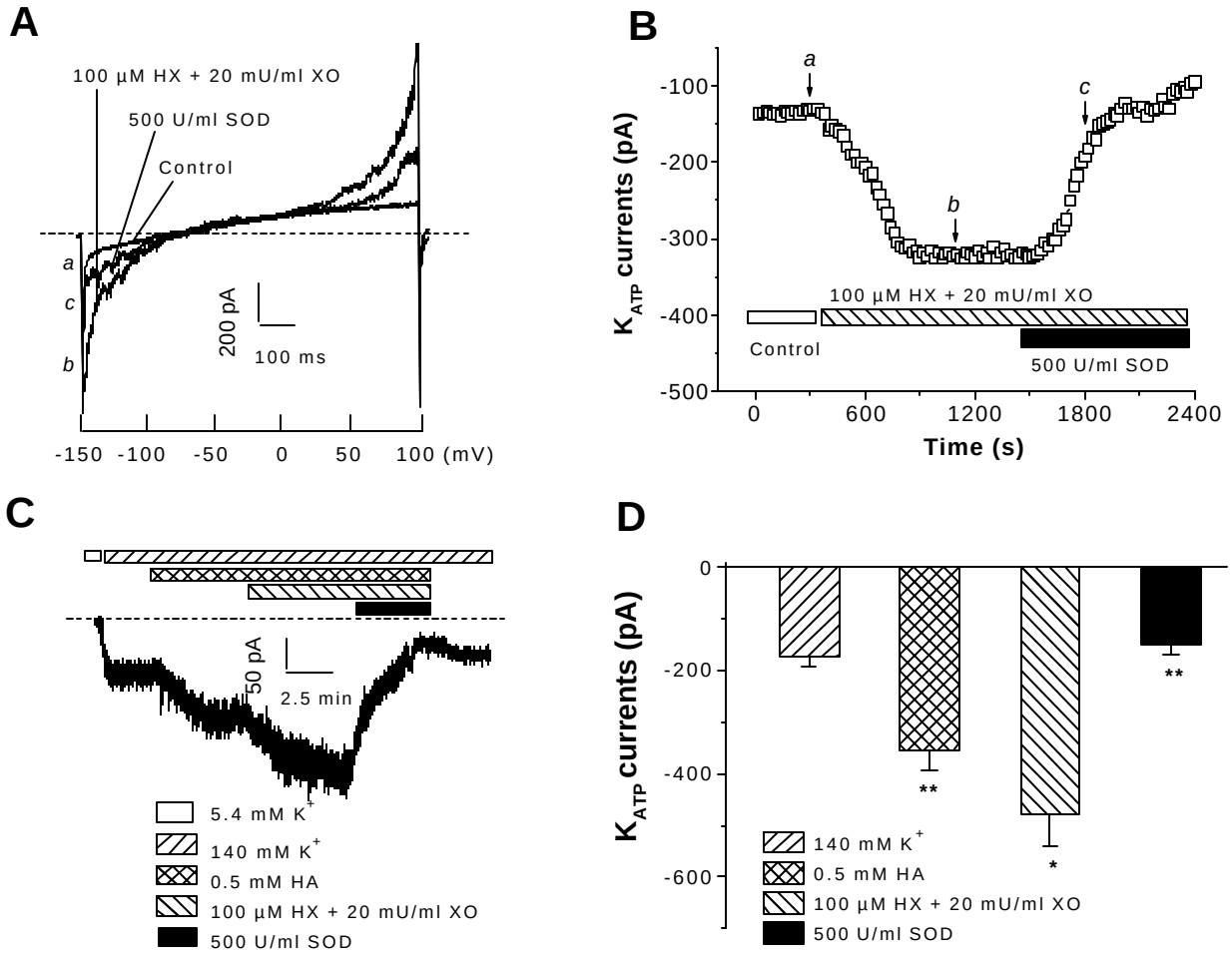


Fig. 29: Effects of hypoxanthine (HX) and xanthine oxidase (XO) on K_{ATP} currents in VSMC. **A.** The original current recordings of the effects of HX/XO (*b*) and SOD (*c*) on basal K_{ATP} currents (*a*) with extracellular 5.4 mM K^+. The ramp pulse was set from –150 mV to +100 mV with holding potential (HP) of –20 mV. The dashed line indicates zero current. **B.** Time-dependent effects of HX/XO (*b*) and SOD (*c*) on basal K_{ATP} currents (*a*). HP= –20 mV and testing potential = –120 mV. **C.** The original current traces showing the effects of 100 µM HX with 20 mU/ml XO and 500 U/ml SOD on hydroxylamine (HA)-stimulated K_{ATP} currents in symmetrical 140 mM K^+ condition. Membrane potential (MP) was held at –60 mV. The current amplitude with slow activation is measured at the maximal value of different treatments for 0.5-1 min. The dashed line indicates zero current. **D.** Summary of the potentiation and suppression of HA-stimulated K_{ATP} currents by HX/XO and SOD, respectively. MP= –60 mV, n=6 for each group. * $p<0.05$ (HX+XO *vs.* 0.5 mM HA), ** $p<0.01$ (0.5 mM HA *vs.* 140 mM K^+; 500 U/ml SOD *vs.* HX+XO).

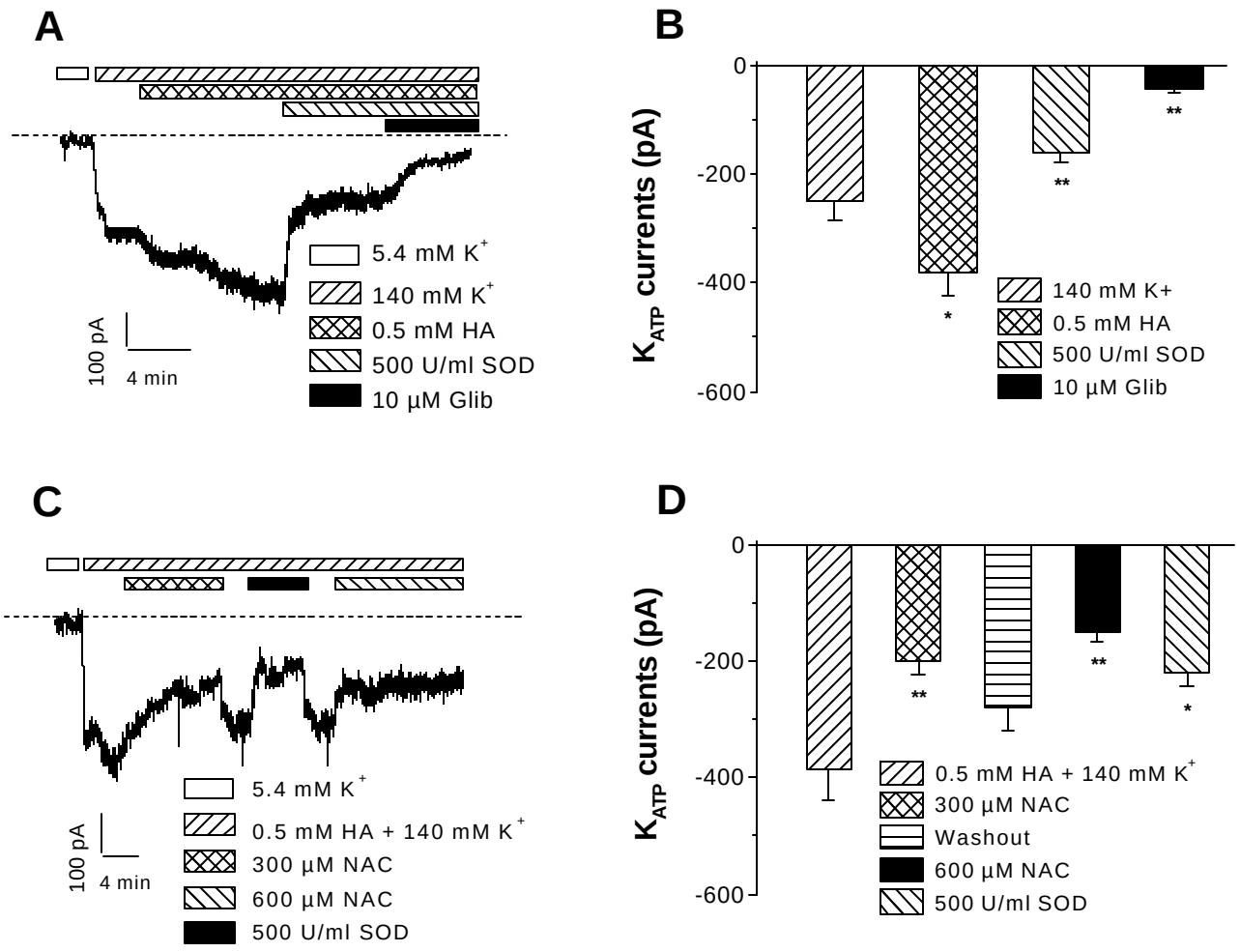


Fig. 30: Effects of free radical scavengers on hydroxylamine (HA)-stimulated K_{ATP} currents in symmetric 140 mM K^+ condition. A. The original current recording of the inhibitory effects of 500 U/ml SOD and 10 µM glibenclamide (Glib) on HA-stimulated K_{ATP} currents. Membrane potential (MP) were held at –60 mV. The current amplitude with slow activation is measured at the maximal value of different treatments for 0.5-1 min. The dashed line indicates zero current. **B.** Summary of the inhibition of HA-stimulated K_{ATP} currents by SOD and Glib. n=4 for each group. * $p<0.05$ (0.5 mM HA *vs.* 140 mM K^+), ** $p<0.01$ (500 U/ml SOD *vs.* 0.5 mM HA; 10 µM Glib *vs.* 500 U/ml SOD). **C.** The original current recording of the reversible inhibition of HA-stimulated K_{ATP} currents by NAC and SOD. MP= –60 mV. The current amplitude with slow activation is measured at the maximal value of different treatments for 0.5-1 min. The dashed line indicates zero current. **D.** Summary of the inhibition of HA-stimulated K_{ATP} currents by NAC and SOD. * $p<0.05$ (500 U/ml SOD *vs.* washout), ** $p<0.01$ (300 µM NAC *vs.* 0.5 mM HA; 600 µM NAC *vs.* washout). n=5 for each group.

4.4.3 Effects of NO donor and cGMP analogue on K_{ATP} currents in VSMC

To examine whether the NO-sGC-cGMP signaling pathway mediated HA effects, a NO donor and a cGMP analogue were used to test K_{ATP} currents. The NO donor, sodium nitroprusside (SNP) at 0.5 mM had no effect on HA-stimulated K_{ATP} currents in symmetrical 140 mM K^+ condition (–293±46 pA *vs.* –284±32 pA, n=5, p>0.05) (Fig. 31A & 31B). With the same recording conditions, the membrane-permeable cGMP analogue, 8-Br-cGMP, failed to affect HA-increased K_{ATP} currents (–370±48 pA *vs.* –345±40 pA, n=5, p>0.05) (Fig. 31C & 31D). Basal K_{ATP} currents were not affected by SNP (–182±23 pA *vs.* –200±30 pA, n=5, p>0.05) or 8-Br-cGMP (–142±21 pA *vs.* –165±23 pA, n=5, p>0.05). However, HA-increased K_{ATP} currents were inhibited completely by glibenclamide at 10 μM, indicating that HA-activated currents are K_{ATP} currents.

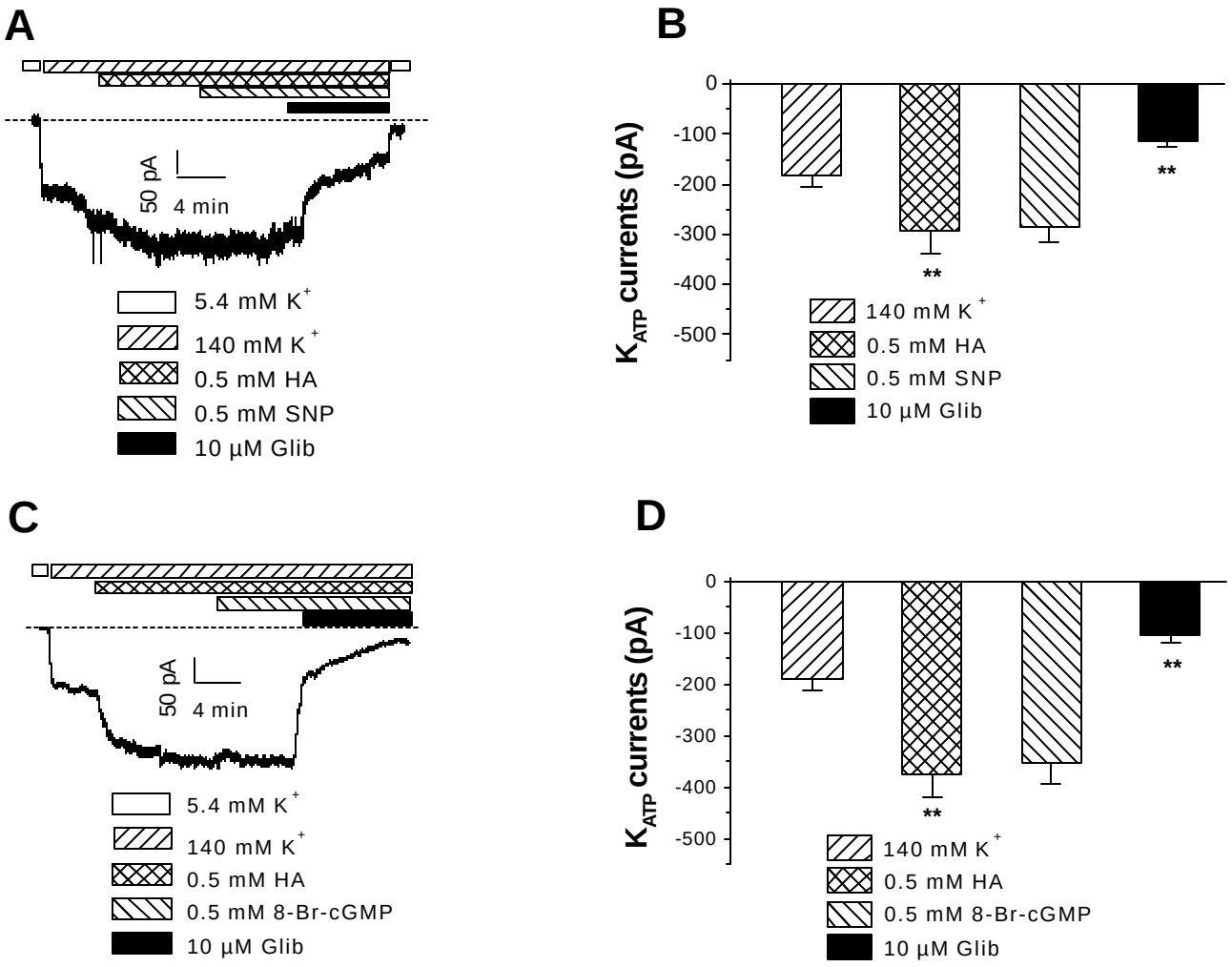


Fig. 31: Effects of sodium nitroprusside (SNP) and 8-Br-cGMP on hydroxylamine (HA)-stimulated K_{ATP} currents in symmetrical 140 mM K^+ condition. A. The original current recording of the effect of SNP and glibenclamide (Glib) on HA-stimulated K_{ATP} currents. Membrane potential (MP) was held at –60 mV. The current amplitude with slow activation is measured at the maximal value of different treatments for 0.5-1 min. The dashed line indicates zero current. **B.** Summary of the effects of SNP and Glib on HA-stimulated K_{ATP} currents. ** $p<0.01$ (0.5 mM HA *vs.* 140 mM K^+; 10 µM Glib *vs.* 0.5 mM SNP). n=5 for each group. **C.** The original current traces of the effect of 8-Br-cGMP and Glib on HA-stimulated K_{ATP} currents. MP= –60 mV. The current amplitude with slow activation is measured at the maximal value of different treatments for 0.5-1 min. The dashed line indicates zero current. **D.** Summary of the effect of 8-Br-cGMP and Glib on HA-stimulated K_{ATP} currents. ** $p<0.01$ (0.5 mM HA *vs.* 140 mM K^+, 10 µM Glib *vs.* 0.5 mM 8-Br-cGMP). n=5 for each group.

5. DISCUSSION

5.1 Summary

The novel findings in this thesis are summarized briefly in the following three sections (5.1.1 to 5.1.3):

5.1.1. Electrophysiological and pharmacological characteristics and functional expression of K_{ATP} channels in rat mesenteric artery VSMC

1) Basal K_{ATP} currents, monitored in the presence of either extracellular 5.4 mM K^+ or symmetrical 140 mM K^+, were activated by MgGDP, pinacidil and diazoxide, and inhibited by glibenclamide. 2) Single K_{ATP} channel activity with unitary channel conductance of 13 pS was also inhibited by glibenclamide and stimulated by diazoxide. 3) The resting membrane potential was depolarized by glibenclamide. 4) In both VSMC and transfected HEK-293 cells, measured reversal potentials at different $[K^+]_o$ match well the calculated K^+ equilibrium potentials, suggesting that these channels are carried by K^+ ions. 5) Transfected HEK-293 cells with Kir6.1 alone exhibited weak inward K^+ currents, which were blocked by external Ba^{2+} and internal Mg^{2+}. 6) Transfection of SUR2B alone also elicited a weak inward current. Whether this subunit represents a functional K_{ATP} channel cannot be concluded. 7) Co-transfection of Kir6.1 with SUR2B produced functional K_{ATP} channel currents, which were activated by MgADP, diazoxide and pinacidil, and inhibited by glibenclamide with an IC_{50} of 1.54±0.2 μM. The co-expressed channel currents were neither stimulated by a low concentration of ATP (0.3

mM), nor inhibited by a high concentration of ATP (3 mM), indicating that Kir6.1/SUR2B was insensitive to ATP inhibition. All these results suggested that Kir6.1/SUR2B might be one isoform of native vascular K_{ATP} channels.

5.1.2. Effects of H_2S on K_{ATP} channels and underlying mechanisms in VSMC

1) Exogenous H_2S enhanced whole-cell K_{ATP} currents and unitary K_{ATP} channel activity and hyperpolarized membrane potentials in rat mesenteric artery VSMC with an EC_{50} value of 116±8.3 μM. H_2S activated K_{ATP} channels by increasing the open probability of single K_{ATP} channels without altering channel conductance. 2) The reduced endogenous H_2S production by CSE inhibitors suppressed K_{ATP} currents. 3) The effects of H_2S on K_{ATP} channels and membrane potentials were not mediated by the cGMP signal pathway. 4) H_2S effects were blocked by CLT, indicating that the activation of K_{ATP} channels by H_2S might be related to the modification of cysteine or methionine residues of channel protein.

5.1.3. Effects of HA on K_{ATP} channels and underlying mechanisms in VSMC

1) HA enhanced reversibly K_{ATP} currents in a concentration-dependent fashion with an EC_{50} of 54±3.4 μM and hyperpolarized the cell membrane of rat mesenteric artery VSMC. 2) HA activated not only the inward component of K_{ATP} currents, but also the IbTX-insensitive outward component of K_{ATP} currents. 3) HA-induced K_{ATP} channel activation and hyperpolarization were blocked by free radical scavengers (SOD and NAC). 4) The free radical generating system (HX/XO) mimicked and potentiated the

effect of HA on K_{ATP} currents, indicating the activation of K_{ATP} channels by O_2^-. 5) SNP and 8-Br-cGMP had no effect on basal and HA-stimulated K_{ATP} currents. Thus, the activation of K_{ATP} channels by HA is likely due to increased free radical generation.

5.2. The electrophysiological and pharmacological characteristics of K_{ATP} channels in VSMC from rat mesenteric artery

5.2.1 The separation and identification of K_{ATP} channels in VSMC

5.2.1.1 Electrophysiological and pharmacological protocols to separate K_{ATP} currents in native cells

In native VSMC, 4 major subtypes of K^+ channels coexist, including K_V, K_{Ca}, K_{ATP} and K_{IR}. While K_V and K_{Ca} channels are outward rectifier K^+ currents, K_{ATP} and Kir channels belong to inward rectifier K^+ channels. Thus, the separation and identification of K_{ATP} currents from other 3 current components in native cells require: i) the design of special voltage protocols for electrophysiological recordings, ii) the manipulation of the ionic compositions of recording solutions, and iii) the utilization of specific pharmacological blockers.

1) The voltage-clamp protocols

Since K_{ATP} currents have a weak inward rectification, the best protocol to record K_{ATP} currents is to choose negative membrane potentials close to the resting potential of cells. Three major voltage protocols were used in this study. For the whole-cell recording, we used step, ramp, and train pulses.

a) Step: The 600 ms test pulses were applied every 10 s with a 10 mV increment from –80 to +70 mV from a holding potential of –60 mV in VSMC (Step I), or from –150 to +80 or +120 mV from a holding potential of –20 mV in HEK-293 cells (Step II). In Step I, the outward component of K_{ATP} current was observed in physiological K^+ conditions with extracellular K^+ at 5.4 mM. The weak point is that the outward K_{ATP} current may be contaminated by Kv and K_{Ca} currents. In Step II, the amplitude of inward K_{ATP} current can be enhanced by an elevation of external K^+ (from 5.4 mM to 40 mM) and by an increase in driving force. The weak point is that the inward current component may be contaminated by Kir currents.

b) Ramp: A 650 ms voltage ramp ranging from –150 mV to +100 mV with a holding potential of –60 mV was used every 10 s. The strong point of this protocol is that both outward and inward components of K_{ATP} currents are observed in the same protocol and in physiological conditions (5.4 mM K^+). The weak point is that this protocol cannot avoid the contamination by Kv, K_{Ca} and Kir of outward and inward currents.

c) Train: This is a protocol to continueously record the membrane current at a fixed membrane potential for a certain period. The membrane current was recorded at a membrane potential of –60 mV in symmetrical 140 mM K^+ condition. This is the best protocol to record K_{ATP} currents, because the negative membrane potential at –60 mV inactivates K_{Ca} and Kv currents and the amplitude of K_{ATP} currents can be enhanced by symmetrical K^+. However, Kir current activation can not be prevented with this protocol except by the addition of a low concentration of Ba^{2+} in the bath solution.

2) The compositions of recording solutions

a) Ca^{2+}-free: To minimize K_{Ca} channel activation, Ca^{2+} was removed from both intra- and extra-cellular solutions. Furthermore, a high concentration (10 mM) and a low concentration (1 mM) of EGTA, a selective Ca^{2+} chelator or quenching agent, were used in the pipette and bath solutions, respectively.

b) ATP+GDP: To maintain K_{ATP} channels in the open state in VSMC, a low concentration of ATP at 0.3 mM was included in the pipette solution. To make basal K_{ATP} currents detectable in the resting state, GDP at 0.5 mM was applied intracellularly to activate vescular K_{ATP} currents in VSMC.

3) The specific pharmacological agents

a) K_{ATP} currents: K_{ATP} currents, instead of Kir currents, are sensitive to a low concentration of glibenclamide at 10 µM, distinguishing K_{ATP} from Kir currents. Pinacidil and diazoxide, specific K_{ATP} channel openers, were used to identify K_{ATP} currents in this preparation (Quayle *et al.*, 1994).

b) Kir currents: Low concentrations of Ba^{2+} at 10-50 µM may block Kir, but not K_{ATP} currents, distinguishing Kir from K_{ATP} currents (Quayle *et al.*, 1993; Edwards *et al.*, 1988; McCarron & Halpern, 1990). However, a high concentration of Ba^{2+} (>100 µM) can inhibit both Kir and K_{ATP} currents (Baiardi *et al.*, 2003).

c) K_{Ca} currents: IbTX at 100 nM was used to test whether K_{Ca} currents were contaminated in the recorded currents.

5.2.1.2 The membrane currents activated by H_2S and HA in VSMC from rat mesenteric artery were mainly conducted by K_{ATP} channels

It is noted that at the beginning of the experiments, Step I protocol (TP= –80-+70 mV, HP= –60 mV, $[K^+]_o$= 5.4 mM) was used to record basal K_{ATP} currents (Fig. 10, 12). Although this protocol demonstrated that glibenclamide inhibited basal K_{ATP} currents in a Ca^{2+}-free bath solution with 5.4 mM K^+, this Step protocol is not suitable to record K_{ATP} currents with a weak inward rectification. Similarly, Step II protocol clearly demonstrated that the inward part of expressed K_{ATP} currents is significantly inhibited or activated by glibenclamide and KCOs, respectively; but the outward part of expressed K_{ATP} currents appears to exhibit a time-dependent inactivation at more positive potentials, suggesting the contamination of Kv in these experiments. This is the reason that the properties of K_{ATP} channels were only analysized using the inward currents. These step protocols were replaced with a train protocol using a membrane potential of −60 mV to record K_{ATP} currents in symmetrical 140 mM K^+ condition (Fig. 11). Some data with H_2S effects (Fig. 20, 25) and most data with HA effects (Fig. 26, 29, 30, 31) on K_{ATP} currents were recorded by using this train protocol. Although the negative membrane potential may prevent the activation of outward K^+ currents like K_{Ca} and Kv, it also elicited the activity of inward rectifier currents (Kir). Thus, it is difficult to rule out the possibility that H_2S- and HA-activated K_{ATP} currents may be contaminated by Kir currents. In fact, it is impossible to completely separate K_{ATP} from Kir currents without using specific pharmacological agents. Of the inward K^+ currents recorded by the train protocols, H_2S- and HA-induced K_{ATP} currents were not completely blocked by glibenclamide, suggesting that other current components may co-exist or the

concentration of glibenclamide is not sufficiently high. In order to confirm that H_2S or HA specifically activates K_{ATP} currents, 1) K_{ATP} currents should be recorded in the presence of 10 μM Ba^{2+} on the extracellular side. The failure of Ba^{2+} to block K^+ currents would exclude the contamination of Kir currents (Quayle *et al.*, 1993). 2) The possible contamination of Cl^- channel currents needs to be excluded by using specific Cl^- channel blockers or the replacement of KCl with K-aspartate. 3) The dose-response curve of glibenclamide needs to be constructed in native VSMC in order to see whether the effects of H_2S or HA can be completely blocked by glibenclamide at sufficient concentration.

Although inward rectifier K^+ currents are known to be expressed in rat mesenteric artery VSMC (Bradley *et al.*, 1999), the recorded K^+ currents under the present conditions were conducted through K_{ATP} channels. The following lines of evidence support this notion. 1) The recorded K^+ current was enhanced by the dialysis of GDP + ATP. NDP-activated K_{ATP} channels are a hallmark of vascular K_{ATP} channels in VSMC. 2) The recorded K^+ current was activated by KCOs like pinacidil and diazoxide and inhibited by glibenclamide. Glibenclamide suppressed not only high K^+-amplified and GDP-activated basal currents, but also H_2S- and HA-stimulated K_{ATP} currents. 3) The recorded K^+ current exhibited a weak inward rectification without voltage-dependence, whereas the classical inward rectifier current was activated by hyperpolarization with strong inward rectification (Quayle *et al.*, 1997). 4) It has been reported that glibenclamide at 10 μM blocked K_{ATP} channels, but had no effect on Kir channels (Quayle *et al.*, 1993; Nelson & Quayle, 1995). In this study, both basal K^+

currents and H_2S- and HA-stimulated K^+ currents were inhibited by 10 μM glibenclamide, supporting a K_{ATP} channel entity. 5) In cells dialyzed with 0.5 mM GDP and 0.3 mM ATP, high K^+-enhanced currents were not blocked by extracellularly applied 10 μM Ba^{2+}, indicating that no Kir channel was activated under these conditions (Quayle *et al*., 1993). 6) Our previous electrophysiological experiments have proved that H_2S failed to affect other subtypes of K^+ channels except for K_{ATP} channels. Our tension studies showed that H_2S-induced vasorelaxation of rat aorta and mesenteric artery bed were only antagonized by glibenclamide, but not IbTX, ChTX, and 4-AP (Zhao *et al.*, 2001; Cheng *et al.*, 2004), while pinacidil produced vasorelaxation similar to H_2S.

5.2.2 The characteristics of single K_{ATP} channels in VSMC

In terms of molecular compositions, K_{ATP} channels are heterogeneous in rat mesenteric artery, in which four channel subunits have been cloned and identified at mRNA levels (Cao *et al.*, 2002). This diversity of the molecular entities of K_{ATP} channels is exemplified in its single channel conductance, ranging from 15-50 pS (small or intermediate conductance) (Davie *et al.*, 1998; Zhang & Bolton, 1995; Wang *et al.*, 2003) to 111-135 pS (large conductance) (Standen *et al.*, 1989; Liu & Zhao, 2000). Our single K_{ATP} channel data in rat mesenteric artery VSMC demonstrated that:

1) K_{ATP} channels were not opened in ATP-free bath solution in inside-out patches. Addition of GDP evoked single-channel activity. Thus, our bath recording solution included 0.3 mM ATP plus 0.5 mM GDP.

2) Single channel conductance is 13 pS in symmetrical 140 mM K^+ recording solution.

3) H_2S stimulated K_{ATP} channel activity through increasing open probability, instead of single channel conductance.

4) KCOs, including pinacidil and diazoxide, opened and glibenclamide blocked basal K_{ATP} channels and H_2S-increased K_{ATP} channels. These results were consistent with the observation that small conductance K_{NDP} channels (20 pS at 60:130 K^+ gradient) open in rat mesenteric artery VSMC in response to GDP, KCOs, and metabolic inhibitors (Zhang & Bolton, 1995).

The heterogeneity of single-channel conductance results from possibly multiple isoforms of channel subunits and the experimental conditions such as excised patch configuration, intra- and extra-cellular K^+ gradients, VSMC types of proximal and distal branches of rat mesenteric artery, etc. Multiple K_{ATP} channel subunits may reconstitute varied channel isoforms, which determined different unitary conductance in vascular tissues. For example, functional Kir6.1/SUR2B channels expressed in mammalian cells closely resembled native K_{NDP} channels in VSMCs. This is especially true with their relatively small unitary conductance (33 pS in symmetrical ~145 mM K^+ solution), bell-shaped relation of ATP actions, ubiquitous MgNDP or KCOs activation, and glibenclamide blockade (Yamada *et al.*, 1997; Satoh *et al.*, 1998; Wang *et al.*, 2003; Thorneloe *et al.*, 2002). In contrast, the heterologously expressed Kir6.2/SUR2B channels in HEK-293 cells elicited functional K_{ATP} channel currents with a unitary conductance of ~80 pS, which were activated by both diazoxide and pinacidil (Isomoto *et al.*, 1996; Fujita & Kurachi, 2000). These channel properties are similar to glibenclamide-sensitive K_{ATP} channels with large conductance (Zhang & Bolton, 1996; Standen *et al.*, 1989; Liu & Zhao, 2000). The diverse range of unitary conductance for smooth muscle K_{ATP} channels may be due to the co-assembly of Kir6.1 and Kir6.2 to

form channels with different combinations of the two pore-forming subunits and their associated SUR2B subunits (Cui *et al.*, 2001; Thorneloe *et al.*, 2002). Expression of Kir6.1 and Kir6.2 with SUR2B yielded a series of channels with distinct levels of unitary conductance ranging from ~35 to ~70 pS. The values at the extremes of this range are consistent with those of Kir6.1/SUR2B and Kir6.2/SUR2B channels at ~35 and ~70 pS, respectively. Three intermediate conductances of channels containing Kir6.1 and Kir6.2 at stoichiometries of 3:1, 2:2, and 1:3 are 43, 55, and 63 pS, respectively (Cui *et al.*, 2001; Thorneloe *et al.*, 2002). This view is supported by expression of tandem Kir6.1-Kir6.2 constructs (to constrain the subunit stoichiometry to 2:2) with SUR2B (Cui *et al.*, 2001) or SUR2A (Kono *et al.*, 2000) which yields channels with a single conductance of ~48 pS, similar to the mid-conductance level observed following co-expression. Therefore, reconstituted channel conductances seem to be determined by the ratio of Kir6.1 to Kir6.2. The higher the Kir6.1 contribution is, the smaller the conductance is. Even if all 4 subunits are Kir6.1, the channel conductance is ~35 pS. The present channel conductance of 13 pS suggests that other Kir subunits may be involved in the formation of such small conductance channels, because multiple members of the Kir family may exist in native VSMC. However, what kinds of Kir subunits are involved need to be further investigated.

In the same rat mesenteric artery and under almost identical conditions (60:120 mM K^+-gradient and negative holding potentials), two different conductances of K_{ATP} channels were found: one at 135 pS (Standen *et al.*, 1989) and another at a level of 20 pS (Zhang & Bolton, 1995). In the case of VSMC derived from rat portal vein, two types of K_{ATP} channels were recorded with different unitary conductances (50 and 22 pS) and various sensitivities to ATP inhibition and NDP activation (Zhang & Bolton, 1996).

Although the K_{ATP} channel conductance reported in this work (13 pS) is different from the one reported in another study (20 pS) (Zhang & Bolton, 1995), it should be noted that they both belong to small-conductance range of K_{ATP} channels in rat mesenteric artery VSMC. The experimental conditions employed for recording K_{ATP} currents are different in these two studies. 1) Single VSMC in this study were dispersed from Sprague-Dawley rat mesenteric arteries in Ca^{2+}-free cell isolation solution by the digestion of collagenase and papain, while Zhang & Bolton (1995) used mice mesenteric arteries to isolate VSMC in low Ca^{2+} solution (10 μM) with the digestion of collagenase and pronase. 2) Symmetrical 140 mM K^+ was used in this study, while a quasi-physiological K^+ gradient ($[K^+]_{o/i}$=60/130) was used in the experiment of Zhang & Bolton (1995). These distinct differences in conditions between different laboratories may explain the differences in single-channel conductance reported by these studies. Furthermore, the unitary channel conductance was affected by analyzing methods such as the direct measurement from the isolated patch recordings and indirect calculation from the amplitude of current noise generated by KCOs in the whole-cell recordings (Criddle *et al.*, 1994).

5.2.3 Contribution of K_{ATP} channels to background K^+ conductance and the setting of resting membrane potentials in mesenteric artery VSMC

It is generally believed that K_{ATP} channels have a very low open probability or remain closed in most VSMC under normal conditions because of high concentration of intracellular ATP. In the absence of K_{ATP} channel openers, the activity of K_{ATP} channels recorded in this study was so low that less than 10% of the recorded patches showed

channel activity. The result was consistent with the reports that the density of K_{ATP} channels in a single arterial smooth muscle was very low, in the range of 300-500 channels/cell (Nelson & Quayle, 1995; Quayle *et al.*, 1997). In order to maintain basal K_{ATP} channel in the open state and in a detectable level, a low concentration of nucleotides (0.5 mM GDP and 0.3 mM ATP) has to be included in the intracellular recording solution. Our results demonstrated that addituion of glibenclamide, in physiological 5.4 mM $[K^+]_o$, inhibited basal K_{ATP} currents in VSMC and depolarized the cell membrane by around 12 mV. These results are in good agreement with previous observations that glibenclamide causes a significant membrane depolarization (5-9 mV) in the resting state of different vascular tissues derived from different species (Clapp & Gurney; 1992; Mishra & Aaronson, 1999; Wilson & Cooper, 1989). Others have shown that glibenclamide also inhibited basal K_{ATP} channels at the resting state with physiological concentrations of intracellular nucleotides (Kubo *et al.*, 1994; Liu & Zhao, 2000; Miyoshi *et al.*, 1992; Wellman *et al.*, 1998; Wang *et al.*, 2003). Thus, K_{ATP} channels in resistant VSMC might be a contributor to the resting K^+ conductance and a regulator of the resting membrane potential. The membrane potential of VSMC is an important regulator of vascular tone by controlling voltage-dependent Ca^{2+} entry (Nelson *et al.*, 1990; Quayle *et al.*, 1997; Jackson 2000). Therefore, K_{ATP} channels might participate in modulating mesenteric artery contractility and contribute to the maintenance of vascular tone in resistance vessels.

5.3. Kir6.1/SUR2B may be one of the isoforms of K_{ATP} channels in rat mesenteric artery VSMC

5.3.1 The expression of Kir6.1 and SUR2B alone in mammalian cell line

In both whole-cell and single-channel recording configuration, Kir6.1 is not expressed in *Xenopus* oocytes (Inagaki *et al.*, 1995a; Gribble *et al.*, 1997), but expressed in mammalian cell lines including COS cells and HEK-293 cells (Inagaki *et al.*, 1995a; Ammala *et al.*, 1996a, 1996b). The possible reason is that functional expression of Kir6.1 may require additional subunits or channel modulators that are endogenously present in HEK-293 cells, but not in oocytes. The single Kir6.1 channel can be closed by 1 mM ATP, opened by 0.1 mM diazoxide, and not changed by sulphonylurea (Inagaki *et al.*, 1995b). In this work, however, internal Mg^{2+} and external Ba^{2+} blocked the whole-cell currents. Other pharmacological properties, such as the sensitivity to sulphonylurea and KCOs, remain to be determined.

Although SUR2B alone has never been expressed in mammalian cells or oocytes, the expression of SUR1 in *Xenopus* oocytes did not result in novel channel activity or confer sulphonylurea sensitivity to the inwardly rectifying K^+ channels such as Kir1.1a, Kir2.1 or Kir3.4 (Aguilar-Bryan *et al.*, 1995). Ammala *et al.* (1996a) demonstrated that SUR1 seemed to act as a regulator of endogenous Kir channels and endow them with sulphonylurea sensitivity, instead of ATP sensitivity although SUR1 did not act as an ion channel by itself when expressed in HEK-293 cells. SUR2B in this study appeared to form functional K^+ currents, but was not conclusive due to the limited amount of pharmacological data.

5.3.2 The co-expression of Kir6.1 and SUR2B in mammalian cell line

The transient expression of SUR2B in the Kir6.1-stably transfected HEK-293 cells generated functional whole-cell K^+ currents with a weak inward rectification. The

co-expressed K^+ currents were significantly activated by pinacidil or diazoxide and dramatically inhibited by glibenclamide although the effects of these pharmacological agents are different to some degree on the co-expressed K_{ATP} currents and their I-V relationships (Cao *et al.*, 2002). For example, pinacidil-increased inward currents were more pronounced than outward currents, while both inward and outward currents were similarly inhibited by glibenclamide. It was noted that glibenclamide inhibited the co-expressed K_{ATP} channels in HEK-293 cells with an IC_{50} of 1.54±0.2 μM, much less sensitive to that in native VSMC from portal vein with IC_{50} of 25 nM (Beech et al., 1993a). This is mainly due to the differences on the dialyzed GDP concentration, channel types, and recorded $[K^+]_o$. The cloned K_{ATP} channel currents in HEK-293 cells transfected with Kir6.1 and SUR2B subunit genes were recoded under 40 mM $[K^+]_o$ condition and with 0.5 mM GDP dialysis; while native K_{ATP} channel currents in VSMC from rabbit portal vein were also recorded under 5.4 mM physiological $[K^+]_o$ and with 10 mM GDP inclusion in pipette solution. These pharmacological results demonstrated that heterologously expressed K^+ currents are K_{ATP} currents because their pharmacological properties shared some similar characteristics with those in native K_{ATP} currents in rat mesenteric artery VSMC.

5.3.3 Heterologously expressed Kir6.1/SUR2B channel closely resembled K_{NDP} channels in VSMC

The heterologous expression of Kir6.1/SUR2B yielded functional K_{ATP} currents in HEK-293 cells with comparable pharmacological properties to the native K_{ATP} currents in rat mesenteric artery VSMC.

1. Similar sensitivity to NDP (ADP or GDP): GDP not only activated microscopic and unitary K_{ATP} currents in native VSMC, but it is also required for the activation of channels evoked by KCOs in both VSMC and HEK-293 cells. This was highlighted by the importance of the inclusion of GDP in the pipette solution to maintain the detectable channel activity. The whole-cell currents of co-expressed Kir6.1/SUR2B channels were activated by ADP in the presence of Mg^{2+}. These indicate that vascular K_{ATP} channels in native VSMC and HEK-293 cells transfected with Kir6.1/SUR2B are sensitive to MgNDP.

2. Insensitivity to ATP inhibition: No channel activity in native VSMC from rat mesenteric artery appeared when the inside-out patch is maintained in ATP-free solution. This observation is consistent with those from rat mesenteric artery and portal vein reported from other laboratories (Zhang & Bolton, 1995, 1996; Beech *et al.*, 1993a; 1993b). A low concentration of ATP (0.3 mM) is dialyzed into cells to keep the channels in an open state in both native VSMC and transfected HEK-293 cells. The reconstituted whole-cell K_{ATP} currents with Kir6.1/SUR2B are not activated by the dialysis of 0.3 mM ATP with time. A high concentration of ATP at 3 mM in the pipette failed to induce significant inhibition in co-expressed K_{ATP} currents. These data indicate both native and cloned K_{ATP} channels exhibit insensitivity to ATP inhibition.

3. Similar pharmacology with respect to glibenclamide and KCOs: Native macroscopic and unitary K_{ATP} currents in VSMC are inhibited by the specific K_{ATP} inhibitor glibenclamide and activated by KCOs (pinacidil and diazoxide) in different $[K^+]_o$. The reconstituted K_{ATP} channels with Kir6.1 and SUR2B were also typically activated by micromolar pinacidil or diazoxide and then significantly inhibited by glibenclamide with an IC_{50} of 1.54±0.2 μM. One could argue that there are some gaps

in the systematic pharmacological profiles, such as the differences in IC_{50} value for glibenclamide or EC_{50} values for pinacidil and diazoxide, between native and reconstituted K_{ATP} channels. However, one can see that both native and cloned K_{ATP} channels share a similar trend in their pharmacological spectrum in both VSMC and HEK-293 cells.

4. Relatively small single-channel conductance: Native K_{ATP} channels presented a small conductance of around 13 pS in symmetrical 140 mM K^+ solution, while heterologously expressed K_{ATP} channels with Kir6.1/SUR2B in the similar recording condition (145 mM external K^+) had a conductance of 33 pS in HEK-293T cells (Yamada *et al.*, 1997). Both channel conductances belong to the small-conductance range, which is one of the characteristics of vascular K_{ATP} channels. Furthermore, Yamada *et al.* (1997) indicated that Kir6.1/SUR2B is not a classical K_{ATP} channel, but closely resembles the K_{NDP} channel in VSMC. The same conclusion cannot be made because the data lack the unitary channel conductance in the reconstituted K_{ATP} channel experiments, which needs to be determined in the future.

Thus, the Kir6.1/SUR2B channels closely resemble the K_{NDP} channels in VSMC, especially in terms of single-channel conductance and ATP sensitivity (Yamada *et al.*, 1997; Satoh *et al.*, 1998; Thorneloe *et al.*, 2002; Wang *et al.*, 2003). The results shown, due to the limited pharmacological data and lack of unitary channel conductance of cloned channels, should be interpreted with caution. Furthermore, because four different K_{ATP} channel subunit genes were cloned from rat mesenteric artery VSMC (Cao *et al.*, 2002), the assembly of Kir6.1/SUR2B is not the sole isoform of K_{ATP} channels in VSMC. There may exist other isoforms like Kir6.2/SUR2B, which has been proposed to represent the molecular composition of glibenclamide-sensitive large-conductance K_{ATP}

channels (111-135 pS) (Standen *et al.*, 1989; Liu & Zhao, 2000). A similar view has been suggested based on observations made for the K_{ATP} channel composition in VSMC isolated from rat mesenteric artery (Isomoto *et al.*, 1996; Fujita & Kurachi, 2000). Thus, the current data available demonstrated that the Kir6.1/SUR2B channel may be one of the isoforms of vascular K_{ATP} channels in rat mesenteric artery VSMC.

5.4. H_2S elicited the activation of K_{ATP} channels and cellular membrane hyperpolarization in VSMC and underlying mechanisms

5.4.1 The effects of endogenous H_2S on K_{ATP} currents in VSMC

In mammalian tissues, CSE and/or CBS cleave L-cysteine to produce H_2S, ammonium and pyruvate. CBS is the predominant H_2S-generating enzyme in brain and nervous system (Kimura, 2000), while CSE is mainly expressed in VSMC (Hosoki *et al.*, 1997; Zhao *et al.*, 2001; Wang, 2002). Results demonstrated for the first time that when CSE activity was inhibited by its specific inhibitors like PPG and βCNA, K_{ATP} currents were reduced in VSMCs. Since the generation of endogenous H_2S from vascular tissues was abolished by PPG (Zhao *et al.*, 2001), it is likely that the inhibition of K_{ATP} currents by PPG and βCNA in this work results from reduced generation of endogenous H_2S due to CSE inhibition. In addition, H_2S dilated vessels like aortic and mesenteric arteries, which was suppressed by PPG perfusion (Zhao *et al.*, 2001; Cheng *et al.*, 2004). Moreover, this H_2S-evoked vasodilation is supported by *in vivo* data that intravenous bolus injection of H_2S transiently decreased blood pressure while intraperitoneal injection of PPG increased blood pressure of rats (Zhao *et al.*, 2001, 2003). These data highlight the cardiovascular function of endogenous H_2S. The

reduction of blood pressure in the former is due to the vasodilatory effect of H_2S; the elevation of blood pressure in the latter is via the inhibition of endogenous production of H_2S. Taken together all these data from both *in vitro* and *in vivo* studies, a major role of H_2S in the maintenance of vascular tone by modulating K_{ATP} channel activity in VSMC has been pointed out.

In the patch-clamp experiments, both PPG and âCNA were applied intracellularly, which directly inhibited K_{ATP} channels in a time-dependent manner and the maximal inhibition occurred in 10-15 min. Furthermore, PPG was reported to cause an irreversible inhibition of CSE enzyme activity *in vitro* (Johnston *et al.*, 1979) and produce an almost complete inhibition of liver CSE activity *ex vivo* (Porter *et al.*, 1996; Uren *et al.*, 1978) when administered to rats. This irreversible inhibition of CSE by PPG was supported by the observation that cysteine, the precursor of H_2S biosynthesis, completely reversed the âCNA-mediated but failed to affect the PPG-mediated increase in contractile response of the ileum to electrical stimulation, reflecting that two inhibitors interact with CSE in different ways, i.e. reversible for â-CNA and irreversible for PPG (Teague *et al.*, 2002).

H_2S at low concentration exerts a range of biological effects as a vasodilator (Wang, 2002) and neurotransmitter (Kimura, 2000); whereas at high concentration or when administered acutely, H_2S becomes toxic via blocking mitochondrial oxidative phosphporylation (Gosselin *et al.*, 1984; Reiffenstein *et al.*, 1992; Dorman *et al.*, 2002). A delicate mechanism *in vivo* exists to maintain the H_2S level within the physiological range, because the rapid oxidation of H_2S in mitochondria (Wang, 2003) may prevent intoxication of cells from the accumulation of the endogenously generated H_2S under physiological conditions. However, what exact concentration of H_2S causes the

physiological or toxicological effects is not entirely clear. H_2S relaxes rat aortic tissues with IC_{50} of 124.7±14.4 μM. In single VSMC, H_2S stimulated K_{ATP} channel activity with EC_{50} of 116±8.3 μM. Thus, the H_2S effect reported in this work belongs to the spectrum of physiological concentration (between 50 μM and 160 μM) of H_2S reported in various biological samples (Hosoki *et al.*, 1997; Zhao *et al.*, 2001; Richardson *et al.*, 2000; Zhang *et al.*, 2003). H_2S at physiological concentration (50 μM) can inhibit cytochrome *c* oxidase, an enzyme critical for oxidative phosphorylation of mitochondrial respiration that lead to the depletion of $[ATP]_i$ (Evans, 1967; Guidotti, 1996; Dorman *et al.*, 2002). It is of concern that the activation of K_{ATP} channels by H_2S in this work could have been due to the indirect depletion of $[ATP]_i$. In these experiments, $[ATP]_i$ was clamped to 0.3 mM and glucose level in the pipette and bath solution were 5 and 10 mM, respectively. These manipulations were sufficient to avoid possible decreases of $[ATP]_i$ levels. Thus, the activation of K_{ATP} channels by H_2S and subsequent hyperpolarization of VSMC is unlikely to result from the reduction of $[ATP]_i$.

5.4.2 H_2S effects on K_{ATP} currents and membrane potentials are independent of cGMP-mediated signalling pathway

The relaxation of VSMC is governed by multiple mechanisms. Different vasodilators have diverse signal transduction pathways. For example, the cGMP-PKG pathway is involved in NO- and CO-induced vasorelaxation (Furchgott & Jothianandan, 1991; Wang *et al.*, 1997; Wang, 1998). Previous work in the laboratory showed that the H_2S-induced relaxation of rat aortic tissues is not mediated by the cGMP pathway (Zhao *et al.*, 2001, 2003; Zhao & Wang, 2002), but endogenous H_2S production was up-regulated by NO in a cGMP-dependent fashion (Zhao *et al.*, 2003). However, whether

the H_2S-induced K_{ATP} channel activation in rat mesenteric artery VSMC is mediated by the cGMP signal pathway has not been defined. In the present work, neither basal K_{ATP} currents nor H_2S-stimulated K_{ATP} currents were affected by extracellularly applied 8-Br-cGMP when membrane current was recorded at −60 mV with symmetrical 140 mM K^+. This result is consistent with other observations that the NO donor SNP and the PKG inhibitor KT5823 had no effect on K_{ATP} currents with the same recording condition as used in this work (Wellman *et al.*, 1998; Quayle *et al.*, 1994). A previous study showed that although ODQ, a specific sGC inhibitor, specifically blocked the vasorelaxation induced by SNP, ODQ did not affect the relaxation of rat aortic tissue induced by H_2S (Zhao *et al.*, 2001). Thus, the vasorelaxant effects of H_2S that was not mediated by the cGMP pathway support the present electrophysiological results. Additionally, low doses (<100 μM) of 8-Br-cGMP and short exposure (<5 min) failed to evoke hyperpolarization of VSMC isolated from rabbit mesenteric arteries (Murphy & Brayden, 1995). The increase of K_{ATP} currents by 8-Br-cGMP was found in cell-attached single channel recording in cultured VSMC from rat thoracic aorta (Kubo *et al.*, 1994), but not in freshly isolated VSMC from resistance mesenteric artery in this work. So there may exist differences in the effects of 8-Br-cGMP on K_{ATP} currents between conduit and resistance artery VSMC. In these experiments, high doses (0.5-2 mM) of 8-Br-cGMP were used to treat cells for more than 15 minutes. This manipulation should rule out the possibility of insufficient increase in intracellular concentration of 8-Br-cGMP. Thus, the modulation of the activity of K_{ATP} channels in VSMC by H_2S is likely independent of the cGMP-mediated pathway.

5.4.3 The role of cysteine residues in the activation of K_{ATP} channels

K_{ATP} channel protein contains critical thiol groups, which may sense changes in metabolism and in the redox potential of cells (Islam *et al.*, 1993; Tricarico *et al.*, 1994, 2000; Linde *et al.*, 1997). During oxidative stress or in the presence of thiol oxidizers, the functional thiol groups of cysteine residues of the K_{ATP} channel protein are switched from the reduced to the oxidized state, altering the activity of K_{ATP} channels (Stadtman, 1992; Lee *et al.*, 1994; Thomas *et al.*, 1995). In inside-out patches of single-channel recordings, thiol-oxidizing agents, such as DTBNP, DTNB, p-CMPS, and thimerosal, induced an inhibition of K_{ATP} channel activity without change in unitary channel conductance in pancreatic â-cells (Islam *et al.*, 1993). Similar results were noted using CRI-G_1 insulin-secreting cells (Lee *et al.*, 1994), rabbit and guinea pig ventricular myocytes (Han *et al.*, 1996; Coetzee *et al.*, 1995). These inhibitory effects were reversed by the addition of disulfide reducing agents DTT or cysteine. These results indicated that thiol-dependent redox mechanisms play a role in the regulation of K_{ATP} channel activity. Further experiments confirmed that thiol-oxidizers act on the cysteine residues of the channel Kir6.0 subunit. Intracellularly applied ñ-CMPS produced an irreversible inhibition on K_{ATP} channels encoded by Kir6.2/SUR1, but not on the mutated channels with C42A mutation of Kir6.2 subunit, indicating that the cysteine residue involved in channel inhibition by ñ-CMPS residues on Kir6.2 subunit and is located at position 42 within the NH_2 terminus of the channel protein (Trapp *et al.*, 1998). In addition, the thiol reducing agents DTT, GSH, NAC, and L-cysteine had no effect on K_{ATP} channel activity when applied alone (Wei & Neumcke, 1989; Tricarico *et al.*, 1994; Caputo *et al.*, 1994; Trapp *et al.*, 1998).

CLT reacts with the exposed thioether group on methionine residues and the sulphydryl group of cysteine residues at pH 7.0-8.5 (Shechter *et al.*, 1975). The covalent modification of different amino acid residues determines the changes in channel activity such as in K_V and K_{Ca} channels. For example, the application of CLT at 2 mM to the cytoplasmic side enhanced single BK_{Ca} channels in inside-out patches by oxidation of methionine residues (Tang *et al.*, 2001). In contrast, the oxidation of cysteine residues suppressed both native and cloned BK_{Ca} channels (DiChiara & Reinhart, 1997; Wang *et al.*, 1997). Bath applied CLT also suppressed the transient outward Kv current (I_{to}) via modification of both methionine and cysteine residues, whereas the enhancement of sustained delayed rectifier Kv currents (I_{dr}) likely results from methionine oxidation alone (Prasad & Goyal, 2004). Extracellular application of a low concentration of CLT (20 µM) irreversibly slowed the inactivation of 4-aminopyridine-sensitive transient outward current (I_{to}) and increased the peak current by 19.3% in rabbit atrial cells, which was partially reversed by subsequent application of 3 mM dithiothreitol (DTT); whereas a high concentration of CLT (100 µM) decreased I_{to} by 22.5%, which was abolished by DTT. These observations revealed that inactivation of I_{to} are susceptible to oxidation of cysteine and methionine residues (Tanaka *et al.*, 1998). Thus, the oxidation of cysteine or together with methionine residues by CLT suppressed K^+ channel activity, while the oxidation of methionine alone enhanced channel activity. In this work, bath perfused CLT abolished H_2S-stimulated K_{ATP} currents, indicating that the oxidation of cysteine and methionine residues abolished the effect of H_2S on K_{ATP} channels. It is still unknown whether H_2S effect can be abolished by the pretreatment of cells with CLT. Whether CLT inhibits basal K_{ATP} currents also needs to be examined.

5.5. HA elicited the activation of K_{ATP} channels and cellular membrane hyperpolarization in VSMC and underlying mechanisms

5.5.1 HA evoked K_{ATP} channel activation and membrane hyperpolarization in VSMC

The HA-induced vasodilation of different vascular tissues has been reported (Huang, 1998; Feelish *et al.*, 1994; DeMaster *et al.*, 1989; Thomas & Ramwell, 1988; Rapoport & Murad, 1984). However, the exact cellular mechanisms underlying vasorelaxant effect of HA has been unclear. It was reported that HA increased the rate of ^{86}Rb outflow from perfused pancreatic islets, which was counteracted by glibenclamide, indicating that K_{ATP} channels were involved in HA-induced inhibition of insulin release (Antoine *et al.*, 1996). HA was also reported to activate voltage-dependent K^+ channels in crustacean skeletal muscle (Hermann & Erxleben, 2001). But a high concentration of HA (10 mM) blocked the inactivating K^+ channels (*Shaker-B*) expressed in *Xenopus* oocytes by an unknown mechanism (Yool, 1994) and depolarized cell membranes by inhibiting K^+ channels (Mongin *et al.*, 1998). Our results demonstrated for the first time that HA stimulated K_{ATP} currents in VSMC and hyperpolarized cell membrane. HA-induced hyperpolarization by K_{ATP} channel activation may close voltage-dependent L-type Ca^{2+} channels and then decrease intracellular free $[Ca^{2+}]_i$, leading to vasorelaxation (Nelson & Quayle, 1995; Quayle *et al.*, 1997).

5.5.2 The activation of K_{ATP} channels by HA may not be involved in the endogenous H_2S generation

Because CBS is a heme-containing protein (Meier *et al.*, 2001) which is a common target of NO and CO, the activity of CBS might be under the influence of both NO and CO (Bruno *et al.*, 2001). HA, an endogenous NO donor, inhibits CBS activity (Braunstein *et al.*, 1971) and then suppresses the production of endogenous H_2S. Thus, HA should inhibit, rather than stimulate K_{ATP} currents in VSMC. On the other hand, since the H_2S-generating enzyme CBS is not expressed in VSMC (Zhao *et al.*, 2001), CBS is not involved in H_2S-induced relaxation of vascular tissues. Taken together, HA-stimulated K_{ATP} channel activity is not likely due to an inhibition of endogenous H_2S production. However, the CSE activity in rat vascular tissues was upregulated by NO released from SNP and SNAP (Zhao *et al.*, 2001), leading to the increase in H_2S production. The NO-induced increase in the H_2S level may involve the cGMP-dependent protein kinase, which in turn stimulates CSE, and/or S-nitrosylation whose potential substrate is the free –SH groups of the 12 cysteines of CSE. However, the possibility that HA-generated NO underlies the activation of K_{ATP} channels by HA can not be ruled out. To this end, whether HA-activated K_{ATP} currents can be inhibited or abolished by PPG remains to be examined.

5.5.3 The NO-sGC-cGMP signaling pathway did not mediate HA-increased K_{ATP} currents

Among the known endogenous K_{ATP} channel modulators is endogenous NO, which activated K_{ATP} channels in cell-attached patches via the activation of soluble guanylyl cyclase (sGC) in cultured VSMC from porcine coronary arteries (Kubo *et al.*, 1994; Miyoshi *et al.*, 1994). Bath-applied atrial natriuretic factor (ANF) and isosorbide dinitrate (ISDN), which are activators of particulate and soluble guanylyl cyclase,

respectively, activated unitary K_{ATP} channel currents. These effects were abolished by methylene blue (an sGC inhibitor) but potentiated by 8-Br-cGMP, suggesting that the effects of ANF and ISDN were mediated by the cGMP pathway (Kubo *et al.*, 1994). At the tissue level, SNP elicited dilation of pial arterioles from anesthetized piglets, which was blocked by a PKG inhibitor (Rp8-Br-cGMP) and a sGC inhibitor (LY83583), indicating that NO primarily elicited its effects via cGMP production (Armstead, 1996; Wilderman & Armstead, 1996). Furthermore, SNP- and 8-Br-cGMP-elicited dilation of newborn pig pial artery was blunted by a K_{ATP} channel antagonist, glibenclamide, indicating that NO and cGMP might interact with K_{ATP} channels (Armstead, 1997). However, SNP- and HA-induced vasorelaxation of rat aortic rings was not affected by glibenclamide, disproving the involvement of K_{ATP} channels in NO-induced vasorelaxation (Huang, 1998). SNP did not increase whole-cell K_{ATP} currents in symmetrical 140 mM K^+ condition, indicating that the activation of NO-sGC-cGMP pathway did not lead to K_{ATP} channel activation (Wellman *et al.*, 1998; Quayle *et al.*, 1994). Therefore, NO effects on K_{ATP} channels in different vascular beds are controversial without clear mechanisms.

Some studies have shown hyperpolarization of smooth muscle by NO via activation of K_{ATP} channels. SNP activated PKG (Lincoln *et al.*, 1994) and produced a glibenclamide-sensitive membrane hyperpolarization in rabbit mesenteric arteries (Murphy & Brayden, 1995). However, other studies in rabbit cerebral and canine coronary arteries failed to demonstrate hyperpolarization induced by exogenous NO (8-30 μM) (Komori *et al.*, 1988; Tare *et al.*, 1990; Himmel *et al.*, 1993). SNP-induced hyperpolarization may result from the cross-activation of PKA by cGMP (Quayle *et al.*, 1997). Only a large amount of NO could produce a hyperpolarizing effect in VSMC

from rat mesenteric artery (Zhao *et al.*, 2000). S-nitroso-N-acetyl-penicillamine (SNAP) at a high concentration (400 μM) caused membrane hyperpolarization, which was reversed by glibenclamide and completely blocked by treatment with Tiron, a scavenger of O_2^-, suggesting that peroxynitrite ($OONO^-$) other than NO exerts the hyperpolarizing effect via the activation of K_{ATP} channels (Zhao *et al.*, 2000).

Our results provide evidence that HA directly activated whole-cell K_{ATP} channels and hyperpolarized the cell membrane, whereas both SNP and 8-Br-cGMP had no effect on basal K_{ATP} currents and HA-stimulated K_{ATP} currents. These findings suggested that the activation of NO-sGC-cGMP signaling pathway did not mediate the K_{ATP} channel activity in rat mesenteric artery VSMC. It is tempting to speculate that HA activated K_{ATP} channels via other mechanism. The yield of free radicals including O_2^- by HA could be one of such mechanisms (Market *et al.*, 1994; Santoian *et al.*, 1993; Vetrovsky *et al.*, 1996).

5.5.4 Free radical generation mainly underlies HA-increased K_{ATP} currents

The modulation of K^+ channel activity by cellular oxidative stress has been recognized as a significant determinant of vascular tone. Under certain conditions, many extracellular ligands generated and/or required free radicals to transmit biological signals to the intracellular milieu as second messengers. Different kinds of free radicals can modify various types of K^+ channels in vascular tissues (Liu & Gutterman, 2002). At the tissue level, O_2^-, H_2O_2 and $OONO^-$ dilated the cerebral vasculature, which was not mediated by sGC activation (Wei *et al.*, 1996). Both H_2O_2 and $OONO^-$ elicited dilation via activating K_{ATP} channels, whereas O_2^- dilated cerebral arterioles by opening K_{Ca} channels (Wei *et al.*, 1996). H_2O_2 induced a glibenclamide-sensitive dose-dependent

dilation in cat cerebral arterioles and rat gracilis skeletal muscle arterioles (Wei *et al.*, 1996; Cseko *et al.*, 2004). $OONO^-$ elicited vasodilation in several vascular beds, including coronary (Liu *et al.*, 1994), renal, mesenteric (Benkusky *et al.*, 1998), and cerebral arteries (Liu *et al.*, 2002; Wei *et al.*, 1996, 1998). Dilation of cerebral and coronary arteries to $OONO^-$ is blocked by glibenclamide, suggesting a role of K_{ATP} channels (Liu *et al.*, 2002; Wei *et al.*, 1996, 1998).

At the cellular level, knowledge about the modulation of K^+ channels by free radicals in single VSMC is still limited. O_2^- produced by xanthine (X)/xanthine oxidase (XO) or high glucose reduced the whole-cell Kv current density in freshly isolated rat coronary VSMC, which was reversed partially by SOD (Liu *et al.*, 2001). However, O_2^- generated by X/XO did not alter significantly the open state probability (NP_o) of K_{Ca} channels (Liu *et al.*, 2002). H_2O_2 activated macroscopic and unitary BK_{Ca} channel currents in porcine coronary arteries via a PLA_2-arachidonic acid signaling cascade (Barlow & White, 1998; Barlow *et al.*, 2000). In isolated coronary arterioles VSMC, the IbTX-sensitive whole-cell K^+ current density was reduced by $OONO^-$ generated from the mixture of SNP with X/XO. $OONO^-$ decreased greatly the NP_o of K_{Ca} channels in inside-out excised patches, contributing to the inhibition of K_{Ca} channel activity (Liu *et al.*, 2002). However, electrophysiological evidence for the effects of free radicals on K_{ATP} channel activity is largely lacking in VSMC. Our results show for the first time the electrophysiological evidence that HA activated K_{ATP} channels in single VSMC from rat mesenteric artery, which was mimicked or potentiated by the free radical generating system HX/XO, and blocked by free radical scavengers like SOD and NAC. If the pretreatment of SOD or NAC does abolish the activation of K_{ATP} currents by HA, this will confirm the involvement of O_2^- in HA effects, which should be tested in the future.

It should be noted that HA in the cytosol is converted into NO and O_2^-, which are likely to form $OONO^-$ (Liu *et al.*, 1994; Huie & Padmaja, 1993; Pryor & Squadrito, 1995). Whether HA-induced K_{ATP} channel activation and vasodilation are linked to $OONO^-$ generation remains to be investigated.

Although hypoxanthine (HX)/XO is widely used as a free radical generating system, direct effects of HX/XO on K^+ channels in single VSMC are rarely reported. When HX is oxidized by XO in the presence of O_2, an electron from the reaction of HX with XO is transferred to O_2 to form O_2^-. The dismutation of O_2^- generates H_2O_2 via cytosolic or mitochondrial SOD. Further oxidation of H_2O_2 leads to highly potent OH^- via the catalysis of transient metals such as ferrous iron (Graf, 1984; Yu, 1994). Thus, HX/XO may generate various reactive species like O_2^-, H_2O_2, and OH^-, which determine different effects of HX/XO, along with species- and tissue-specific differences in various vascular beds. Application of HX/XO together with $FeCl_3$ to pial artery *in vivo* resulted in attenuated vasodilatation induced by K_{ATP} channel agonists (cromakalim and calcitonin gene-related peptide), NO donors (SNP and SNAP), and 8-Br-cGMP (Armstead, 1999). From these results, however, it cannot be concluded that O_2^- inhibits K_{ATP} channels in VSMC. Changes in diameter of pial artery *in vivo* are under influences of many vasoactive substances with multiple mechanisms. Blocking a common downstream cellular event by HX/XO would not only inhibit the vasodilatory effect of K_{ATP} channel agonists, but also that of many other vasodilators which may not interact with K_{ATP} channels at all. Direct effects of HX/XO on the basal diameter of pial arteries were not examined. Electrophysiological evidence for the effect of HX/XO on K_{ATP} channels in VSMC of pial arteries was also unavailable. In the present work, direct electrophysiological recording of K_{ATP} channel currents was carried out on isolated

VSMC from rat mesenteric artery. Both electrophysiological and pharmacological results demonstrated that the HX/XO reaction in fact activated K_{ATP} channels in single VSMC. This effect is likely mediated by O_2^- since HX/XO-activated K_{ATP} currents were blocked by SOD. In summary, HA-induced K_{ATP} channel activation and the resultant hyperpolarization in VSMC may underlie HA-induced vasorelaxation via enhanced production of free radicals.

6. CONCLUSIONS AND SIGNIFICANCE

6.1. K_{ATP} channels in VSMC are important modulatory targets

K_{ATP} channels are involved in many cellular responses by coupling cell metabolism to the membrane potential. K_{ATP} channels in resistance VSMC from rat mesenteric arteries contribute to the background K^+ conductance and to the setting of resting membrane potential, playing an important role in the regulation of vascular tone, peripheral vessel resistance, and arterial blood pressure. K_{ATP} channels are unique in that two structurally distinct proteins, Kir6.x and SUR, are both required for their functional expression. The heterologous expression of Kir6.1 and SUR2B genes in mammalian cell lines forms functional channels and elicits whole-cell K_{ATP} currents, which share similar biophysical and pharmacological characteristics of native K_{ATP} channels in VSMC. Co-expressed K_{ATP} channels represent vascular K_{NDP} channels and constitute one isoform of native vascular K_{ATP} channels. SUR2B, as a regulatory subunit that endows pore-forming Kir6.1 with sensitivity to sulphonylurea, KCOs, and MgNDP, determines that K_{ATP} channels become important targets for endogenous metabolic regulators and exogenous therapeutic drugs.

6.2. H_2S and HA modulated channel activity with different mechanisms

Both exogenously applied and endogenously generated H_2S cause K_{ATP} channel activation, indicating that H_2S action appears to be direct and needs no intermediates. Exogenous H_2S gas or H_2S released from its donor, NaHS, activated K_{ATP} channels in VSMC and hyperpolarized the cell membrane. The inhibition of endogenous H_2S production by PPG suppressed K_{ATP} currents. The activation of K_{ATP} channels by H_2S was independent of cGMP-mediated signal pathway (Fig. 32). On the other hand, free radicals like NO oxidized –SH groups of K_{ATP} channels via N-nitrosylation, leading to channel opening. However, exogenous and endogenous NO donors had different effects, indicating that NO action may be indirect and need intermediates. The exogenous NO donor SNP failed to activate K_{ATP} currents, while the endogenous NO donor HA stimulated K_{ATP} channels and hyperpolarized membrane potentials. This difference is likely due to the formation of O_2^- from HA metabolism in the cytosol. However, SNP did not generate O_2^-. Furthermore, HA-activated K_{ATP} channel currents were blocked by the free radical scavengers SOD and NAC and mimicked by the free radical generating system HX/XO. This indicates that HA-generated O_2^- is an activator of K_{ATP} channels in VSMC (Fig. 32). HA-stimulated K_{ATP} currents are not due to the generation of NO or the production of endogenous H_2S.

Taken together, K_{ATP} channels in VSMC serve as the regulatory targets of H_2S and HA. These two endogenous substances modulated K_{ATP} channels with different mechanisms. H_2S acted on the K_{ATP} channel protein by a cGMP-independent mechanism, while HA oxidized K_{ATP} channel protein via the formation of O_2^-, altering

the stabilization of channel protein structure and leading to the activation of K_{ATP} channels.

6.3 Significance

Our results provided evidence that endogenously generated H_2S contributes significantly to the regulation of K_{ATP} channels in VSMC. The importance of H_2S as a gasotransmitter in homeostatic control of cardiovascular function has been adequately established by the data shown in the present thesis. By establishing the mechanism by which H_2S directly interacts with K_{ATP} channels, the contribution of K_{ATP} channels to the regulation of cardiovascular function, including its regulation of vascular tone and blood pressure, can be better understood. On the other hand, the abnormal production and metabolism of endogenous H_2S might be related to the pathogenesis of cardiovascular diseases such as hypertension, atherosclerosis, stroke, and diabetes. Patients with homocystinuria exhibit significantly elevated concentrations of homocysteine and are accompanied by a reduced circulating level of H_2S; whereas Down's syndrome with elevated CBS expression and low plasma homocysteine may be coupled to abnormally high H_2S levels. Homocysteine causes endothelial cell injury and cell detachment that initiates the development of arteriosclerosis. The altered level of circulating H_2S may also affect the structure and function of VSMC, thus joining homocysteine as a compounding pathogenetic factor for arteriosclerotic cerebro- and cardio-vascular diseases. Thus, new therapeutic strategies to alleviate cardio- and cerebro-vascular diseases can be devised via affecting endogenous H_2S production.

Traditionally, HA was used as a simple analog of clofilium, a class III cardiac antiarrhythmic compound, which prolongs the action potential by inactivating Shaker K^+ channels in cardiomyocytes (Yool, 1994). The present work demonstrated that HA activated K_{ATP} channels in VSMCs via the generation of O_2^-, rather than NO and H_2S. HA hyperpolarized single VSMC membrane via activating K_{ATP} channels, which may underlie the vasodilatory response evoked by HA in different vascular beds. These results shed more light on new mechanisms in support of vascular actions of HA. Understanding the mechanisms by which HA-generated O_2^- affects K_{ATP} channels may direct new therapeutic approaches in overcoming vascular dysfunctions and for the treatment of cardiovascular diseases.

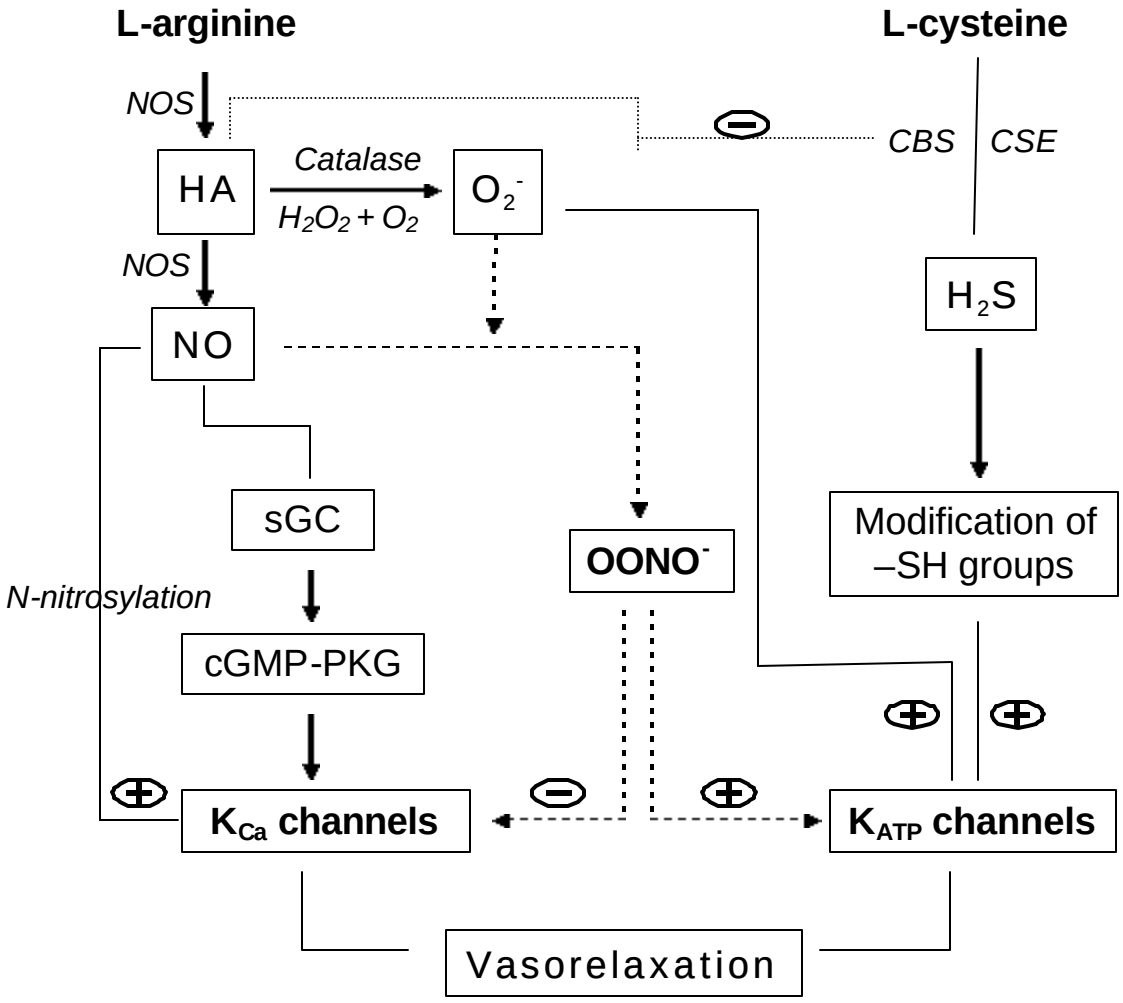


Fig. 32: The hypothesized mechanisms of H_2S and HA actions in VSMC. NO activates K_{Ca} channels via activating sGC-cGMP-PKG signalling pathway; whereas H_2S action is modulated via a cGMP-independent mechanism. NO activates directly K_{Ca} channels through N-nitrosylation and K_{ATP} channels likely via the formation of free radicals $ONOO^-$. H_2S activates K_{ATP} channels mainly via an action of –SH groups of channel protein. Hydroxylamine (HA) activates K_{ATP} channels likely via the generation of O_2^- or the formation of $OONO^-$.

7.0 FUTURE DIRECTIONS

To extend and expand our findings reported in this thesis, the following key experiments are proposed in the future.

7.1. To further characterize the electrophysiological and pharmacological features of co-expressed K_{ATP} channels (Kir6.1/SUR2B or SUR2B alone) in HEK-293 cells

So far, it has not been confirmed that Kir6.1/SUR2B represents the molecular composition of vascular K_{ATP} channels in rat mesenteric arteries. This is due to the lack of the unitary channel conductance of the co-expressed K_{ATP} channels with Kir6.1 and SUR2B and lack of information on their detailed pharmacological sensitivity to K_{ATP} channel openers (pinacidil and diazoxide) and a specific inhibitor (glibenclamide). Therefore, the first future study will use the single-channel recording technique to determine the single channel conductance of the co-expressed K_{ATP} channels with Kir6.1/SUR2B. Then, the whole-cell K_{ATP} currents in co-expressed K_{ATP} channels will be tested by pinacidil, diazoxide and glibenclamide, respectively, to be consistent with previous data generated by the whole-cell patch-clamp technique. The EC_{50} of pinacidil and diazoxide and the IC_{50} of glibenclamide will be calculated and compared. The pharmacological properties of the expressed channels with SUR2B alone also need to be examined in order to increase the number of cells tested by diazoxide and glibenclamide.

All these additional experiments will facilitate the understanding of the molecular basis of vascular K_{ATP} channels.

7.2. To examine the effects of H_2S on expressed K_{ATP} channels (Kir6.1 alone or Kir6.1/SUR2B) and on mutated co-expressed K_{ATP} channels (Kir6.1/SUR2B) in HEK-293 cells

H_2S activated whole-cell and single-channel K_{ATP} currents in native VSMC. However, molecular mechanisms of the interaction of H_2S and K_{ATP} channels have been largely unclear. Whether H_2S will stimulate co-expressed K_{ATP} channels with Kir6.1/SUR2B or Kir6.2/SUR2B isoforms in HEK-293 cells is an intriguing question. To explore which subunit of K_{ATP} channels is the target for H_2S, the effects of H_2S on expressed K_{ATP} channels in HEK-293 cells transfected by either Kir6.1 subunit alone or a combination of Kir6.1/SUR2B subunits will be examined. If H_2S can stimulate K_{ATP} currents in coexpressed channels with Kir6.1/SUR2B, rather than Kir6.1 alone, this will indicate that H_2S interacts with SUR2B subunits.

H_2S action appears to relate to the cysteine residues of K_{ATP} channels. Whether H_2S breaks the disulfide bond or modifies free sulphydryl groups is unclear. The cysteine scanning mutagenesis technique will be used to replace cysteine residues with serine residues of Kir6.1 and/or SUR2B subunit of coexpressed K_{ATP} channels encoded with Kir6.1/SUR2B. Then, the effects of H_2S on mutated K_{ATP} channels will be tested. If H_2S effect on a mutated K_{ATP} channel with specific cysteine replacement is abolished, this cysteine residue as the target of H_2S can be extrapolated.

7.3. To determine the contribution of the H_2S-generating enzyme (CSE) to endogenous H_2S production in CSE-knockout mice

H_2S is endogenously synthesized in the enzymatic reaction catalyzed by CBS and CSE with tissue-specific expression. However, most currently available enzyme inhibitors are not membrane-permeable, which significantly impedes their application under physiological conditions and thus affects the exploration of the physiological and pathological role of endogenous H_2S. It is imperative to develop novel alternative avenues such as pharmacological or genomic manipulation of H_2S production. A heterozygous deficiency of CBS mice has been established. Deficient CBS expression causes hyperhomocystinemia with low levels of H_2S, leading to premature occlusive arterial diseases like atherosclerosis and thrombotic complications, whereas elevated CBS expression in infants with Down' syndrome couple to abnormally high H_2S levels, causing sudden death. The transgenic animal model with CSE deletion will be needed to establish the contribution of this enzyme to endogenous H_2S levels in vascular tissues. It would be exciting to examine the alterations in cardiovascular functions in CSE knockout mice if CSE knockout mice are generated and become commercially available.

7.4. To examine whether $OONO^-$ is involved in HA-induced K_{ATP} channel activation and resultant hyperpolarization in VSMC

HA was suggested to activate K_{ATP} channels likely via the generation of O_2^-. HA is known to be metabolized into NO and O_2^- in the cytosol, by which NO and O_2^- may combine with each other to form $OONO^-$ (Fig. 32). Whether HA stimulates K_{ATP} currents through the formation of $OONO^-$ should be tested. The $OONO^-$ scavenger, uric

acid, will be employed to test the change of K_{ATP} currents induced by HA. If HA-evoked increases in K_{ATP} currents would be blocked by uric acid, this would indicate that $OONO^-$ is involved in HA-activated K_{ATP} currents. If activation of K_{ATP} currents induced by HA were abolished after pretreatment with superoxide scavengers SOD or Tiron, this would indicate that $OONO^-$ is indeed involved in HA-induced K_{ATP} channel activation. To examine whether $OONO^-$ directly activates K_{ATP} channels in VSMC, the synthesized $OONO^-$ solution or the simultaneous addition of NO donor (SNP) with O_2^- generating system (HX/XO) will be used to perfuse cells. If K_{ATP} currents were increased under such conditions, it would imply that $OONO^-$ is most likely involved since HX/XO also activates K_{ATP} channels via O_2^- generation. If the increased K_{ATP} currents are suppressed by uric acid, this would confirm the involvement of $OONO^-$.

7.5. To examine the effects of HA on the expressed K_{ATP} channels with Kir6.1/SUR2B or Kir6.1 alone in HEK-293 cells

HA stimulated K_{ATP} channels in native VSMC. Whether HA activates cloned K_{ATP} channel remains to be elucidated. The expressed K_{ATP} channels with Kir6.1 or SUR2B alone or in combination in HEK-293 cells will be tested. If HA activates the co-expressed K_{ATP} channels, this may provide direct evidence that HA activated K_{ATP} channels in VSMCs. If HA only activates the co-expressed K_{ATP} channels, but not Kir6.1 channel alone, this would indicate that HA activates K_{ATP} channel via targeting the SUR subunit. If the activation of the cloned K_{ATP} channel by HA is abolished by –SH oxidizers like DTNB and CLT, this would indicate that HA activates K_{ATP} channel via the oxidation of free –SH groups by O_2^-. All these experiments will definitely provide

greater insights into the molecular mechanisms by which HA-derived ROS modulate K_{ATP} channel function in vascular tissues.

7.6. To explore the effects of H_2S and HA on K_{ATP} channels in vascular ECs.

The endothelium plays an important role in the regulation of vascular tone by secreting both vasoconstrictors (endothelin) and vasodilators (prostacyclin PGI_2, NO, and EDHF etc.). Because endothelial cells (ECs) do not express voltage-dependent Ca^{2+} channels, Ca^{2+} influxes, following receptor activation by vasoactive agents, may be facilitated by cell hyperpolarization mediated by the activation of K^+ conductances. The presence of K_{ATP} channels have been demonstrated in freshly dissociated ECs from rabbit aorta (Katnik & Adams, 1997), rat and bovine pulmonary microvasculature (Chatterjee *et al.*, 2003), and rat aorta and brain microvasculature (Janigro *et al.*, 1993). Whether K_{ATP} channel activation contributes to Ca^{2+} entry in ECs is unclear. H_2S and HA induced the relaxation of mesenteric artery bed (MAB) and aoric tissues and stimulate K_{ATP} channel activity in single VSMC from both arteries. Furthermore, removal of endothelium or co-application of charybdotoxin and apamin to endothelium-intact MAB significantly reduced the vasorelaxation effects of H_2S (Cheng *et al.*, 2004). These results suggest that H_2S may have two targets: K_{ATP} channels in VSMC and ChTX/apamin-sensitive K_{Ca} channels in vascular ECs, the target of EDHF. The activation of these two types of channels by H_2S would compound to hyperpolarize VSMC, leading to vasorelaxation. Since HA is a putative intermediate of NO synthesis from L-arginine in ECs, whether HA itself serves as EDHF is unknown. Whether H_2S and HA act on K_{ATP} channels in single vascular EC is an intriguing question. Future

studies on the effects of H_2S or HA on electrophysiological properties of resistance artery ECs would help better understand the endothelium-dependent regulation of vascular smooth muscle tone.

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9. APPENDIX

Before the present thesis is going into print, some manuscripts and abstracts produced from thesis data have been published or submitted for publication in the peer-reviewed journals or communicated in the national and international conferences. These publications are listed below.

9.1 Articles published or submitted in peer-reviewed journals

1) Cao K, Tang G, Hu D & Wang R. 2002. Molecular basis of ATP-sensitive K^+ channels in rat vascular smooth muscles. *Biochem Biophys Res Commun* **296**, 463-9

2) Cheng Y, Ndisang JF, Tang G, Cao K & Wang R. 2004. Hydrogen sulfide induced relaxation of resistance mesenteric artery beds of rats. *Am J Physiol Heart Circ Physiol*. **287**, H2316-H2323

3. Tang G, Wu L and Wang R. 2004. Stimulation of K_{ATP} channels in vascular smooth muscle cells by hydrogen sulfide and the underlying mechanism (in preparation)

4. Tang G, Wu L and Wang R. 2004. The effects of hydroxylamine on K_{ATP} channels in vascular smooth muscle and underlying mechanisms, *Molecular Pharmacology* (revision)

9.2 Abstracts published in refereed journals or communicated in conferences

1) Tang G, Wu L and Wang R: The breakage of disulfide bonds of K_{ATP} channels – A novel mechanism for the physiological roles of hydrogen sulfide, **National Research Forum for Young Investigators in Circulatory and Respiratory Health (*YI Forum*)**, organized by CIHR Institute of Circulatory and Respiratory Health and Partners, Winnipeg, Manitoba, Canada, May 6-9, 2004

2) Tang G, Wu L and Wang R: The effects of hydroxylamine on K_{ATP} channels and underlying mechanisms in vascular smooth muscle cells, **Experimental Biology 2003 *(EB '03)*: Translating the Genome**, organized by Federation of American Societies for Experimental Biology (FASEB), San Diego, California, USA, April 11-15, 2003, and also be presented at **BioContact Quebec 2003 Symposium**, organized by CIHR and Biopharmaceutical Partners, Quebec City, Quebec, Canada, October 1-4, 2003

3) Tang G and Wang R: The effects of endogenous H_2S on K_{ATP} channels and membrane potentials in VSMCs from rat mesenteric arteries, Can J Cardiol 2002, 18 (Suppl B), P108 B. **55th Canadian Cardiovascular Congress/23rd Annual Meeting of Canadian Hypertension Society**, organized by Canadian Cardiovascular Society and Canadian Hypertension Society, Edmonton, Alberta, Canada, October 26-29, 2002

4) Tang G and Wang R: The effects of H_2S on K_{ATP} channels and the underlying mechanisms in resistance artery smooth muscle cells, *Cardiovascular Drugs & Therapy* Vol. 16, Suppl. 1, P86. **11th International Congress on Cardiovascular Pharmacotherapy** (**ISCP 2002**), organized by International Cardiovascular Pharmacotherapy Society, Montreal, Quebec, Canada, May 18-21, 2002.

5) Tang G, Cao K and Wang R: Heterologous coexpression of cloned vascular smooth muscle K_{ATP} channel subunit genes (rvKir6.1/rvSUR2B) in HEK-293 cells, **9th Annual Life Sciences Student Research Day**, organized by College of Medicine, University of Saskatchewan, Saskatoon, January 18, 2002

6) Tang G, Cao K and Wang R: Characteristics of cloned cDNA encoding vascular smooth muscle K_{ATP} channels expressed in HEK-293 cells, **The International Meeting of Frontier on Hypertension Research** (***FHR-2001***) organized by International Hypertension League, Weifang, China, June 6-9, 2001

7) Tang G, Cao K and Wang R: Functional expression and pharmacology of cloned cDNA encoding vascular smooth muscle K_{ATP} channels in HEK-293 cells, **8th Annual Life Sciences Student Research Day**, organized by the College of Medicine, University of Saskatchewan, Saskatoon, January 19, 2001